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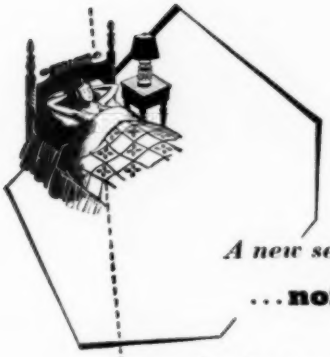

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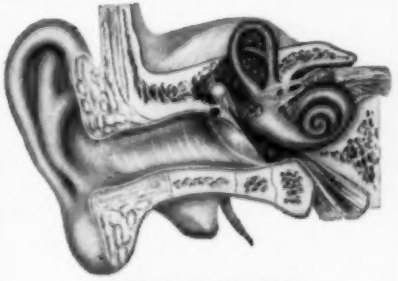

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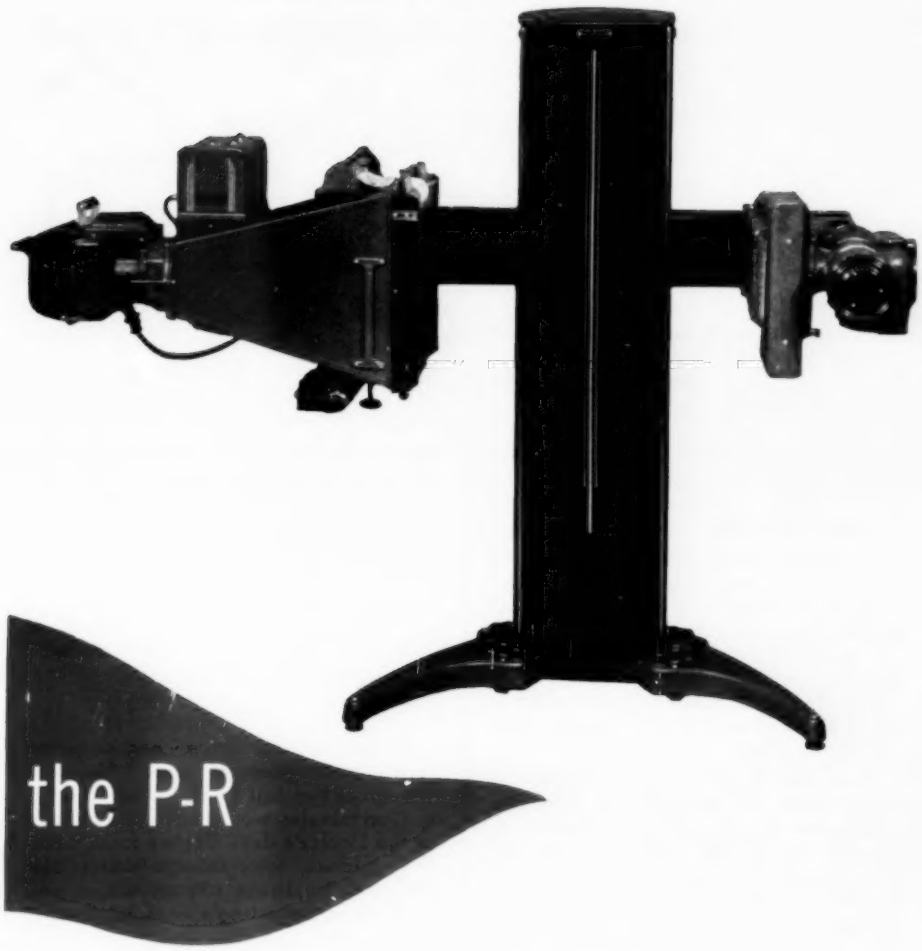

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LATE NONTUBERCULOUS COMPLICATIONS OF CALCIFIED HILUS LYMPH NODES[1, 2]

JEROME R. HEAD AND CHESTER W. MOEN

(Received for publication June 16, 1948)

INTRODUCTION

In most instances, primary tuberculosis of the lungs is a benign infection. The focus in the lung and the larger centers in the hilus and mediastinal lymph nodes pass through their acute stages without producing important symptoms or complications, and usually the permanent calcifications which result are matters of pathological and roentgenological interest, rather than of clinical importance. This is not always the case. Guersant, Le Blond (1), and Laennec (2) reported numerous cases in which acutely inflamed tuberculous nodes had occluded and ruptured into the bronchi, lung, trachea, and esophagus, producing extensive and fatal disease. Parrot (3). Hutinee (4), and Kuss (5) noted this, and that the primary focus in the lung could progress to caseation and excavation and to the development of widespread tuberculous pneumonia. They observed also that the infection could pass beyond the nodes to involve the thoracic duct and produce miliary tuberculosis. More recently, Kent (6) has pointed out that the entity commonly called epituberculous pneumonia is in reality an atelectasis caused by enlarged hilus lymph nodes, and Lightwood and Wilson (7), Hughes and Simpson (8), and Huizinga (9) have shown that a transient atelectasis so produced during the acute stages of primary tuberculosis is a common cause of chronic nontuberculous bronchiectasis.

These are all complications of the acute phases of the primary tuberculous complex. The subject of this paper is the late nontuberculous complications of healed or quiescent calcified tuberculous nodes at the root of the lung. As has already been stated, the acute primary infection in these nodes usually progresses to spontaneous arrest and to calcification. In most cases this is an encapsulation and an arrest and not a cure of the infection. Tubercle bacilli remain alive in the nodes for many years and extension from this source is a common cause of reinfection bronchial and parenchymal tuberculosis. That in itself is a large and important aspect of the pathogenesis of tuberculosis. It does not fall within the scope of this paper, however, which is concerned only with the late nontuberculous activation of these calcareous nodes. However long these calcifications may have been present, and in spite of the absence in them of live tubercle bacilli, they can, and frequently do, become secondarily infected and enlarge to press upon, occlude and perforate the bronchi and even the esophagus with the production of recurring pneumonia, atelectasis, bronchi-

[1] From the Department of Surgery, Northwestern University, Chicago, Illinois.

[2] Presented before the Medical Section, as part of the symposium on *Surgery-Trends-Late Results*, at the 44th Annual Meeting of the National Tuberculosis Association, New York, New York, June 16, 1948.

ectasis, and lung abscess. Such activation is frequently associated with hemoptysis and, what is more dramatic, with the expectoration of particles of calcium. The condition can be the cause of nearly every type of pulmonary infection and frequently produces a clinical picture scarcely distinguishable from bronchial carcinoma. For these reasons, it has seemed worthwhile to recall attention to it and to emphasize that it is common and not rare, and that it can be present without being signalized by the expectoration of calcium.

HISTORY

Nontuberculous disease of the lungs associated with the expectoration of calcareous particles has been noted since the earliest times. Aristotle, in the course of dissections of animals carried out at the instigation of Alexander the Great, noted that the lungs frequently showed rock-like indurations similar to those found in the spleen and kidney (4). Aretaeus of Cappadocia and Galen reported cases of cough with expectoration of stones (4). Galen conjectured that they were formed in the bronchi by the drying of thick secretions. Alexander of Tralles reported the history of a man who expectorated calcareous concretions so large that they made a noise when they struck the ground. Paulus Aegineta saw the expectoration of many stony concretions associated with hemoptysis. Poulalion, who wrote a treatise on the subject in 1891 and who is the source of much of this historical review and bibliography, mentions similar cases reported by various ancient authors (4).

In 1600 Schenck (4) reviewed the subject and cited the case of Dr. Oswald Gabelhower who was cured of chronic lung disease by the expectoration of a calculus. He mentioned many similar cases reported by various physicians.

In 1720, Richard Morton (10) in his classification of types of phthisis included that produced by calculi. He wrote:

> I have often observed chalky stones bred in the lungs, which, when they have been angular and disturbed with the shaking of the lungs, are wont to tear the tender substance of those parts; from whence have arisen a violent and dry cough and a horrible pain in the breast, like that in Pleurisy and Peripneumony; sometimes also an hemoptysis with a considerable flux of blood and from thence ulcers, with the usual signs of a consumption of the lungs. Therefore, when these horrid pains happen with an hemoptysis about the beginning of a consumption, we may justly suspect it to be a consumption from stones in the lungs, although we cannot pronounce anything certain of this thing until the stones have been coughed up. Usually these stones get into the windpipe by which they are coughed up, and that seldom without a great flux of blood. But if the stones are smooth they may occasion only a dry cough and something of an oppressing weight in the breast. But when angular, and sharp, they break and tear the lungs and cause pain, spitting of blood, ulcers, and a consumption itself, as I have hinted.

In 1746, Boerhaave (11) wrote:

> The celebrated botanist, Vaillant, spit up 400 stones from his lungs before death, though they were all of them indeed small and round. He was afflicted with an asthma from a calculous matter collected in the vesicles of the lungs. I have seen asthmas of the worst kind, in which the patient has been tortured with incessant coughing without intermission, 'til after some weeks they have brought up calculi from the windpipe, and the respiration has been free for some time, 'til more calculi were again formed. I have seen other patients who have kept by them large cups full of these stones which had been brought up from the lungs. Such people as these die with a spitting of blood; for the tender fabric of the lungs is destroyed by the violent coughing used to bring up these stones.

In 1761, Morgagni (12) described the clinical and pathological findings in such cases. In a letter to William Bromfield of London he wrote in detail of the respiratory troubles resulting from the presence of concretions in the lungs. Poulalion states: "He established the distinction between deposits in the lungs, in the bronchial glands and those formed within the bronchi from the condensation of secretions." (4).

It was not until the early nineteenth century that calcifications within the lung and bronchial nodes were recognized as being the result of tuberculosis. Bayle (13) considered calcareous phthisis as distinct from tuberculous phthisis. Broussais (4) and Laennec (2) were the first to insist on their tuberculous origin. This confusion arose undoubtedly from the fact that the expectoration of calcium was sometimes associated with active tuberculosis and sometimes with acute or chronic nontuberculous pulmonary infections.

In 1891, Poulalion (4) included in his thesis a section on what he termed "pseudo-tuberculose calculaire." At this early date he mentions the fact that actinomycosis, cladothricosis, and other mycotic infections can produce pulmonary calcifications.

Since that time, as before, there have appeared numerous reports of individual cases or of groups of cases. These appear in articles by Bickel and Grunmach (14), Pritchard (15), Pendergrass (16), Pratten (17), Stivelman (18), Lloyd (19), Van Ordstrand (20), Anderson and Mackey (21), Arthur Elliott (22), Myers (23), Tinney and Moersch (24), and Maytum and Vinson (25). Most of these are reports of one or of a few cases. Van Ordstrand (20) described thirteen cases observed between 1900 and 1930, and Tinney and Moersch (24) twenty-eight cases collected from the files of the Mayo Clinic.

ETIOLOGY

Just why a healed and encapsulated calcified node, after remaining stationary and innocuous for many years, should suddenly begin to cause trouble is uncertain. In the cases under consideration, it is not a reactivation of a latent tuberculosis, for tubercle bacilli are not found and the resulting parenchymal infection is not tuberculous. One must conclude, I believe, that the calcium, like any other foreign body, invites secondary infection and that when such infection occurs it persists until the foreign body has been removed or extruded. Such late secondary infection develops about calcium deposits in the lung parenchyma and about layers of calcium deposited on the pleura.

There are four routes by which bacteria could reach calcified hilus lymph nodes: (*1*) by direct extension from infection and ulceration of the bronchial wall; (*2*) by extension from contiguous lung tissue; (*3*) by way of the blood stream; and (*4*) by way of the lymphatics. Which is the usual route is a matter for speculation. It seems probable that the nodes, not being completely involved, continue to function as lymph nodes and that bacteria reach them by way of the lymphatics from infections of the lung parenchyma. It is certain that in many instances uninfected calcified nodes produce a permanent narrowing of a contiguous bronchus and that this narrowing predisposes to recurring infection in the distal bronchi and parenchyma. Such a node is more exposed to infection than one which does not drain a repeatedly reinfected lobe.

ANATOMICAL OBSERVATIONS

The regularity in position of the hilar lymph nodes was pointed out by H. P. Nelson (26) in 1932. This consistency in the relation of the hilar nodes to the

bronchial tree and pulmonary vessels was also noted in our series of fifty cadaver dissections. From the cases reported in the literature and in this paper, it is noted that the most frequent sites of stricture and perforation by calcified nodes were in those portions of the bronchi where lymph nodes were normally found.

The important bronchial nodes present were as follows:

In the right hilus were three large nodes which were present in 100 per cent of the cases. The largest of these was seen from the posterior aspect of the hilus and was present in the angle of the bifurcation of the trachea, the greater part of the node lying in contact with the medial surface of the right main stem bronchus. Another large node was present between the under surface of the upper lobe bronchus and the main stem bronchus. It partially encircled the upper lobe bronchus in all cases. The third node was located in the angle formed by the middle and lower lobe bronchi. This was the smallest of the three nodes and averaged one cm. in diameter. Another node in the right hilus which is often regarded as important was located in the angle formed by the trachea and the right main stem bronchus. However, in our series, this node was present in only 20 per cent of the cases and averaged but 7.6 cm. in diameter.

In the left hilus there were also four principal bronchial nodes. Only one of these was present in 100 per cent of the cases. This node was located between the lingular bronchus and the lower lobe bronchus. From the anterior aspect, in the angle formed by the left main stem bronchus and the left upper lobe bronchus was a large node which was present in 80 per cent of the cases. From the posterior aspect of the left hilus, a large node was seen on the upper border of the main stem bronchus in 80 per cent of the cases. Another large node was seen on the inferior surface of the main stem bronchus in 70 per cent of the cases. The last two nodes were present together in the same specimen in 44 per cent of the cases.

SITE OF PERFORATION

With the anatomical position of the bronchial nodes in mind, the opinions of various authors regarding the most frequent site of perforation will be given.

M. Paunz (27) stated that there was a prevalence of perforation on the right side on the medial wall of the right main bronchus, *i.e.*, the interbifurcation node was the greatest offender.

B. Schick (28) mentioned that the right main bronchus was most frequently affected by stones because of the increased frequency of the primary complex on the right.

S. Engel (29) stated that the right bifurcation node and the right tracheal node were most frequently affected and that the majority of perforations occurred in this area.

C. Petot (30) demonstrated anatomically that the lymph nodes to be most frequently enlarged and to exert pressure were those found surrounding the right stem bronchus in the area of the bronchus to the right middle lobe.

In 1944 O. Auerbach (31) reported his examination of the bronchial tree in 1,656 autopsies in which he found 22 perforations. In his series he found that perforations occurred most frequently in the area of the carina, but that the

distribution was fairly equal between the trachea, the right and the left main stem bronchi.

The cases reported in the literature reveal almost equal distribution of involvement among the bronchi to all the lobes, with the exception of the right upper lobe bronchus which was affected the most (30 per cent of all cases), and the lingular bronchus which was not affected at all.

In our cases the frequency of involvement was distributed among the right upper, the left upper, and the middle lobe bronchi, respectively.

CLINICAL PICTURE

The symptoms and physical and roentgenographic findings can simulate every type of acute and chronic bronchial and pulmonary disease. Basically, all of the findings are caused by bronchial ulceration, narrowing, and occlusion. Hemorrhage may be the only symptom. *There may be merely recurring attacks of bronchopneumonia in the same lobe.* The cough associated with this is apt to be severe and spasmodic. Blood streaking or frank hemorrhage are common. During the acute stage a persistent wheeze localized over the affected lobe is a common and suggestive sign. This may disappear after the expectoration of calcium. Lung abscess and empyema may complicate the lung infections.

Recurring attacks of acute atelectasis produce a dramatic picture. Poulalion (4) speaks of sudden attacks of bronchial colic associated with dyspnea, fever, and relieved by the expectoration of calcium. Morton comments on the "horrid pain" in such cases (10). The atelectasis may persist and all types of chronic pulmonary suppuration may develop.

Attacks tend to be recurrent. They usually stop finally and one can assume that this occurs when all of the calcium has been extruded, yet Boerhaave's patient expectorated 400 stones over a period of many years (11).

In one case in the present series, the calcified gland at the bifurcation ruptured into the right lower lobe bronchus and also into the esophagus, producing an esophageal bronchial fistula.

The most dramatic symptom is the expectoration of stones. These may vary in size from small and sand-like grains to particles one cm. in diameter. It is surprising how often the patient fails to be impressed with this finding sufficiently to report it to his physician. For this reason it is necessary to question all patients on this specific point. When so questioned, one of our patients produced from his wallet an envelope containing a dozen or more stones. He had never shown them or mentioned them to any of the many doctors who had attended him.

The roentgenographic findings are as variable as the bronchial and pulmonary conditions which are produced. One must suspect the condition and remember that calcifications at the root of a lobe may be responsible for disease in the parenchyma. Laminography may give important information in revealing a calcified node impinging upon a bronchus.

Bronchoscopy rarely shows more than granulation tissue and bronchial narrowing. Manipulation often produces important bleeding.

TREATMENT

The treatment depends upon the nature, the severity, and the chronicity or recurrence of the symptoms. In many instances the calcium is extruded and expectorated spontaneously, the fistula in the bronchus closes without producing important narrowing, and the infection in the lung clears up. There may be only one such episode or they may recur at indefinite intervals.

In such cases, and in all cases at first, medicinal treatment of the parenchymal infection is all that is indicated. Lung abscess or empyema may develop and may require surgical treatment. In one case in this series an esophageal bronchial fistula produced a threatening situation. That it closed following endoscopic cauterization of the openings seems fortuitous. Such a complication today would be an indication for surgical intervention.

Bronchoscopic removal of the calcium is difficult and dangerous. The nodes and bronchi are contiguous to large pulmonary vessels. Bleeding is apt to be severe and may be fatal.

If the infection persists or recurs with bothersome frequency, lobectomy is indicated. If the offending node is not infected at the time of the operation and the bronchus is not greatly narrowed, removal of the node may be feasible and sufficient.

CASE REPORTS

Case 1. A male, age 38, was first admitted to the hospital on December 19, 1946, complaining of a nonproductive cough and wheeze for three weeks. There were flecks of blood in his sputum and on three occasions he had coughed up small solid particles "like chalk." On the day of his admission, he coughed up some more particles which relieved his cough considerably. Physical examination revealed no significant findings. His temperature was 99.4°F. The total erythrocyte count was 4.7 million per cu. mm. and the total leukocyte count was 9,650 per cu. mm. Roentgenographic examination revealed no abnormalities except for calcified deposits in the right hilus. The bronchoscopic examination revealed a white mass arising from the posterior wall of the bronchus at the origin of the right upper lobe bronchus. This was interpreted as calcium. The manipulation of this mass by forceps produced a brisk hemorrhage and bronchoscopy was discontinued. The patient continued to wheeze and cough for another week and then suddenly developed a chill and fever of 103°, accompanied by a leukocytosis of 23,000 cells per cu. mm. Physical examination revealed increased breath sounds and some scattered rales over the area of the right lower lobe. Roentgenographic examination revealed a light, hazy, bronchopneumonic type of infiltrate in the lower portion of the right lung. The following night (January 1, 1947) he had another chill and fever of 103°. At the same time he had a severe coughing attack and brought up a large calcified plaque (figure 1). Immediately after coughing up the calcium, the patient noted a disappearance of his wheeze. A daily remission of temperature followed and it was normal on January 7, 1947. The chest roentgenogram on this date was also normal.

Case 2: (Figure 2). A man, age 34, began to have frequent colds and to cough up foul-smelling sputum and small flecks of blood in September 1944. At the same time he began to wheeze and cough up small pieces of stone from time to time. On November 19, 1944, he had a chill and fever of 103°F. and was admitted to an Army hospital. He was coughing up a cupful of pus daily and was then given penicillin and sulfonamide therapy. He improved clinically but there was little change on the chest roentgenogram. Again in April 1945, he

FIG. 1 (Case 1). Stones expectorated by this patient. The large mass was an amalgam of pieces of calcium and blood clot.

FIG. 2 (Case 2). Small lung abscess peripheral to calcified lymph node. This patient coughed up approximately one-half ounce of small stones over the course of several months.

had another episode of chills and fever with expectoration of much foul pus. Another course of penicillin was given and again he improved clinically but without much change on the roentgenogram. On June 20, 1945, the patient came into the office with a jar containing a great number of stones he had coughed up a few days before. By the first week in July 1945, his chest roentgenogram was almost normal and his wheeze had diminished a great

deal, although he was yet coughing up small particles of calcium. Bronchoscopy at this time revealed a friable mass of tissue on the medial aspect of the right main bronchus at the level of the right middle lobe bronchial orifice. The lower lobe orifice was stenotic but smooth.

Case 3: (Figure 3). A woman, age 43, was admitted to the hospital February 26, 1944, complaining of severe coughing attacks and wheezing for the past three days. She stated that she had had similar attacks on several occasions since 1941. Sometimes the attacks had been accompanied with chills and fever. On admission the total erythrocyte and leukocyte counts were normal. The chest roentgenogram appeared similar to the one taken in 1941,

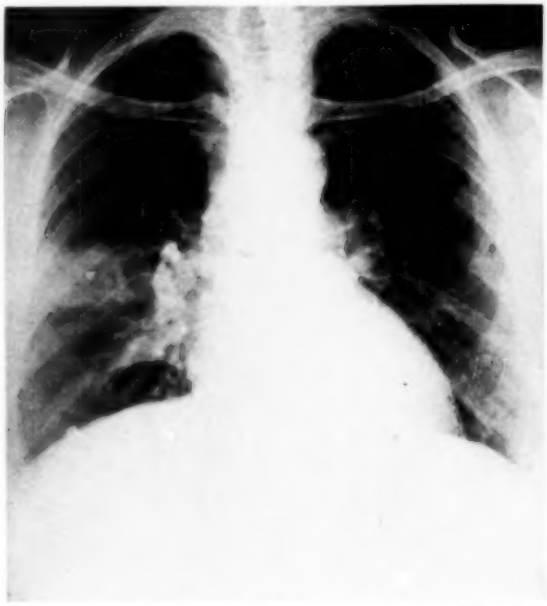

FIG. 3 (Case 3). Area of pneumonia and abscess distal to eroding calcified nodes.

which revealed a large primary complex in the hilus of the right lung with parenchymal infection about it. Bronchoscopy revealed granulation tissue and constriction at the beginning of the right main stem bronchus at the level of the carina. Two weeks later the patient coughed up several pieces of calcium which gave her considerable relief from cough and wheezing. Again during the first week in April 1944, she coughed up some more calcium. After this her roentgenogram showed considerable clearing.

Case 4: (Figures 4A and B). A man, age 36, had empyema and pneumonia in 1933. In June 1943, he developed a cough, had pain in his left chest, dyspnea, and a fever of 102°F. The roentgenographic examination revealed several calcified nodes at the hilus of both lungs and an area of parenchymal infiltration radiating outward from the left hilus to the infraclavicular area. Repeated sputum examinations, cultures, and guinea pig inoculations were negative for tubercle bacilli. However, the patient was advised to be on bed rest. He stayed in bed until October 1943, with little improvement symptomatically or on the chest

roentgenogram. In the last week of October, he had a severe coughing attack and brought up several large pieces of calcium. Roentgenographic examination on November 15 showed almost complete clearing of the lesion (figure 4 B). Since then he has been completely well.

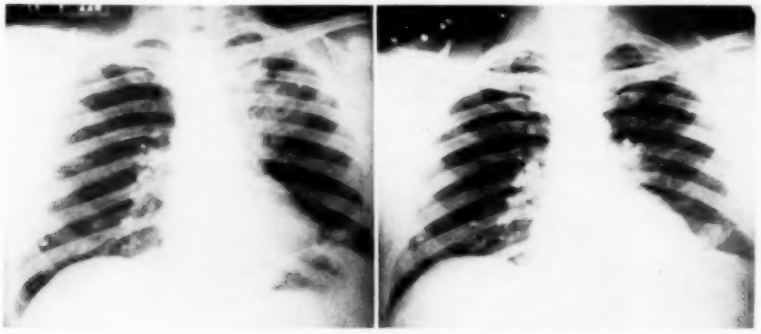

FIG. 4 (Case 4)

A. (Left) Bronchopneumonia in left upper lobe peripheral to calcified gland eroding into the bronchus.

B. (Right) After expectoration of stones.

FIG. 5 (Case 5). Infiltration in left upper lobe which cleared following expectoration of particles of calcium.

Case 5: (Figure 5). Three weeks prior to admission, a 42 year old man began having daily episodes of hemoptysis, small in amount, not associated with any pain. He had no fever on admission and the total leukocyte count was normal. The chest roentgenogram revealed an increased density along the left side of the mediastinum just above the hilus. Within this area of density were several calcium deposits. Extending outward and upward from this area were some fibrous strands which brought up the question of an old tuberculous or malignant process. Bronchoscopy showed a bleeding area at the orifice of the upper lobe

bronchus. Bronchial secretions were negative for tumor cells. One week after admission, the patient coughed up several pieces of calcium. Two days later the area of increased density had disappeared from the chest roentgenogram.

Case 6: (Figure 6). A 49 year old white woman had a severe grinding pain behind the xiphoid process five weeks prior to admission. At the same time she developed a cough which was deep-seated and paroxysmal. Two weeks later she had an identical attack and expectorated some pus and several small pieces of calcium. One hour prior to admission she had another coughing attack with substernal pain during which she had an hemoptysis which filled an emesis basin. After hospitalization the cough subsided considerably.

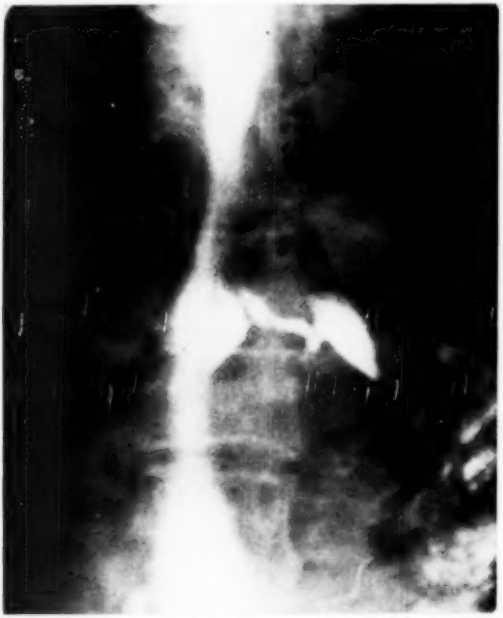

FIG. 6 (Case 6). Broncho-esophageal fistula secondary to erosion of calcified node into the bronchus and esophagus. After expectoration of calcium and cauterization of fistulous openings through the bronchoscope and esophagoscope, patient made a complete recovery.

Roentgenographic examination of the chest revealed no abnormalities. After ten days the patient began to have difficulty in swallowing and coughed severely after eating and drinking. Barium studies of the esophagus revealed a definite tracheo-esophageal fistula approximately at the level of the bifurcation of the trachea (figure 6). Through the bronchoscope, a nipple-like protrusion in the posterior wall of the right main bronchus immediately below the bifurcation was seen. Esophagoscopy revealed a fistula along the right lateral wall, 30 cm. from the teeth. Gastrostomy was done. Two weeks later the patient developed a lung abscess which was drained satisfactorily. After three months the fistulous tract had healed and the gastrostomy tube was withdrawn. For the past four years the patient has been well.

Case 7: (Figures 7 A and B). A woman, age 25, was admitted to the Veterans Hospital in May 1947, complaining of a chronic, productive cough of two years' duration. The cough

had gradually become more severe and the sputum more purulent. On five separate occasions the patient had expectorated a teaspoonful of bright blood. The first hemoptysis occurred in October 1945, and the last in April 1947. In October 1945, the chest roentgenogram was negative, but a second examination in February 1946 revealed a "possible lung abscess in the right middle lobe." At this time the patient had no fever, but a course of penicillin and sulfonamide was given. There was no improvement either clinically or on the chest roentgenogram. In November 1946, she expectorated a piece of calcium "one-half inch in diameter" along with a tablespoonful of blood. During her recent admission the patient had no fever. The physical examination was essentially negative and tubercle bacilli were not found in the sputum. Roentgenographic examination revealed an atelectatic middle lobe and a large calcified node near the origin of the middle lobe bronchus (figures 7 A and B). Bronchoscopy revealed a stenotic middle lobe orifice. Examination of material obtained by biopsy of this area was negative for tumor tissue. After three months there was spontaneous clearing of the middle lobe atelectasis.

FIG. 7 (Case 7)

A. (Left) Atelectasis of the middle lobe caused by erosion of calcified node into the bronchus.

B. (Right) Oblique film on same patient.

Case 8: (Figures 8 A and B). A 40 year old man was admitted to the Veterans Hospital August 5, 1947, complaining of a chronic, productive cough since October 1946. At that time he had been admitted to a hospital with acute symptoms of chills, fever, cough, and chest pains. A diagnosis of pneumonia was made and he had been treated with penicillin and sulfadiazine. His acute symptoms abated but the chronic, productive cough persisted. Because of this cough he came to the Veterans Hospital for treatment. The significant physical findings were confined to the right chest posteriorly. In this area there was increase in tactile fremitus, impairment of resonance, and depression of breath sounds. The chest roentgenogram revealed an area of increased density at the right base and numerous calcified glands in the region of the right hilus (figure 8 A). This was interpreted as atelectasis produced by the calcified nodes. Bronchoscopy revealed a narrowing of the right middle lobe bronchus, and a study of the bronchial secretions was negative for tumor cells. The bronchogram showed an abrupt obstruction of the right middle lobe bronchus with pooling of the lipiodol at this point (figure 8 B). Since the presence of carcinoma could not be excluded, thoracotomy was performed. The right middle lobe was completely atelec-

FIG. 8 (Case 8)

A. (Left) Atelectasis of the middle lobe caused by erosion of calcified node into the bronchus.

B. (Right) Lateral film of same patient after injection of iodized oil showing the occluded bronchus.

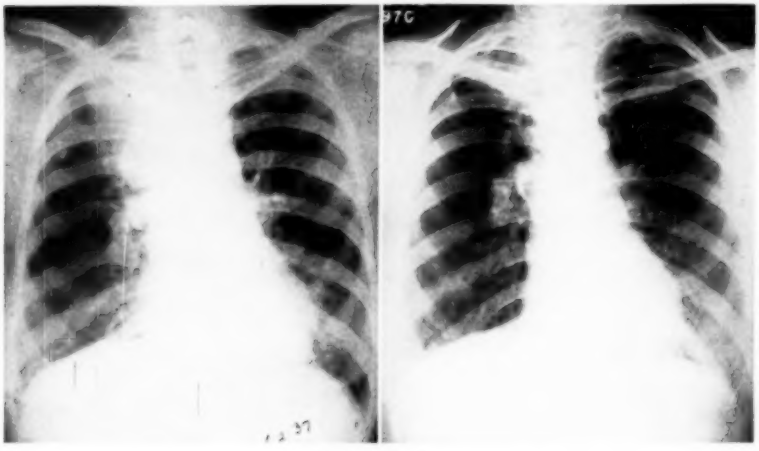

FIG. 9 (Case 9)

A. (Left) Atelectasis of right upper lobe caused by perforation of calcified nodes into the bronchus.

B. (Right) Roentgenogram of same patient after expectoration of numerous particles of calcium.

tatic and many calcified nodes were adherent to the origin of the middle lobe bronchus. Lobectomy was performed with difficulty and microscopic examination of the bronchus revealed only granulation tissue. The postoperative course was uneventful.

Case 9: (Figures 9 A and B). A woman, age 40, was first seen in a California clinic on June 1, 1937. She complained of chest pain of four months' duration, cough for one month, and weight loss of 20 pounds in six months. The physical examination was essentially negative. The roentgenographic examination revealed a tumor mass at the right superior mediastinum which appeared to be a node. In addition, there was an area of increased density involving the upper third of the right lung. Bronchoscopy revealed the presence of creamy pus exuding from a compressed right upper lobe bronchus. Examination of the smear revealed gram-positive cocci but no acid-fast organisms. The serologic test for syphilis was also negative. The patient was given fifteen courses of deep X-ray therapy and in September 1937 the dense area in the right upper lobe had decreased considerably so that numerous calcium deposits could be seen in the right hilus. The cough and pain subsided. The patient entered the Northwestern clinic in October 1937, complaining that her chest pain and cough had reappeared. Physical examination revealed rhonchi and wheezes over the right upper lobe. The chest roentgenogram was essentially the same as the last one taken. The patient was again treated with deep X-ray therapy and cough medicines. The cough persisted until December 1938, when she coughed up a matchbox full of stones. This relieved her cough for a few months and then it recurred. She coughed up stones again in 1940, 1943, and in May 1944. Her last visit to the clinic was in August 1944. At that time her cough was almost gone and bronchoscopy revealed a normal bronchial tree.

Case 10: A man, age 51, was admitted to the Veterans Hospital complaining of chronic cough with the expectoration of blood and pus for the past year. On several occasions he had expectorated calcium plaques together with considerable quantities of blood. Roentgenographic examination revealed an increased density and numerous calcium deposits in the upper part of the left hilus. Bronchoscopy revealed a fixation of the left bronchus with some distortion and roughening of the lateral wall above the upper lobe orifice. Examination of the material obtained by biopsy of this area revealed tumor cells. One week after admission he coughed up more than a washbasin full of blood and died. At autopsy a bronchiogenic carcinoma was found in the left upper lobe.

SUMMARY

A series of cases illustrating various nontuberculous complications of calcified hilus lymph nodes is presented. The symptoms and the clinical and roentgenologic findings of this entity can simulate every type of acute and chronic bronchial and pulmonary disease. Basically, all of the findings are caused by bronchial ulceration, narrowing, and occlusion. The choice of treatment is decided on the basis of the nature and extent of the accompanying pulmonary injection.

SUMARIO

Complicaciones No Tuberculosas Tardías de los Ganglios Linfáticos Calcificados del Hilio

Preséntase una serie de casos que demuestran varias complicaciones no tuberculosas de los ganglios linfáticos calcificados del hilio. La semiología y los hallazgos clínicos y roentgenológicos pueden simular cuanta variedad existe de afección bronquial y pulmonar, ya aguda o crónica. En el fondo, todos los hallazgos son ocasionados por úlcera, estenosis y oclusión bronquial. La

selección del tratamiento se basa en la naturaleza y extensión de la infección pulmonar concomitante.

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OBSERVATIONS ON LOWER LOBE DISEASE IN PULMONARY TUBERCULOSIS[1]

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INTRODUCTION

Some sixty years ago Kidd (1) and Fowler (2) recognized that the lower lobe could be the site of the initial reinfection lesion in pulmonary tuberculosis. Study in the ensuing years confirmed their observations. Particularly in the last two decades, judging by the increasing frequency of reports on this subject, there has been a growing interest in this phase of tuberculosis.

Recently the authors had a rather large cross section of pulmonary tuberculosis available, during a period of service at a former Army General Hospital[2]. With an interest in lower lobe disease, a study of the case files of the chest service at this hospital covering a three year period[3] was done. Its purpose was to observe the incidence and results of treatment in an all male group of military age as compared to the reports in the literature.

Definition: By pure anatomical definition, lower lobe would refer only to the posterior inferior lobes of the lung. However, at this institution the term was used to include the right middle lobe as well. From a practical point of view, disease in the right middle lobe presents problems comparable to those associated with basilar disease. Realizing that the lingula could be considered the left "middle lobe," the authors kept this anatomical factor in mind. In reviewing the following cases, no instance could be found where the initial lesion was demonstrated in this portion of the lung field.

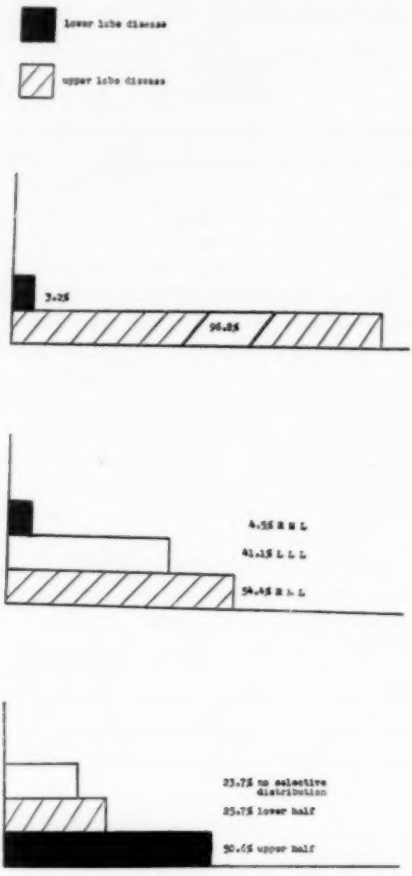
The scope of this study has been confined to those cases with definite roentgenological evidence that the initial lesion occurred in the right middle lobe, or the right or left lower lobes. Likewise, in the cases considered from the standpoint of the results of treatment, the predominant involvement was in the lower lobe, even though serial films showed dissemination of a lesser degree to the upper lung fields.

OBSERVATIONS

General incidence: A review was made of 2,784 successive cases of tuberculosis in which the diagnosis had been definitely established at the time of discharge or transfer from Bruns General Hospital. All of the patients were males. In 2,777 cases the tuberculosis was considered to be of reinfection type; in the remainder the infection was in the form of a primary complex. Guided by the foregoing definition, 90 cases of lower lobe disease were found. This represented 3.2 per cent of the entire group (figure 1). Eighty-five infections were classified as reinfection type, or 3.1 per cent of the total reinfection group.

[1] From the Bruns General Hospital, Santa Fe, New Mexico.
[2] Bruns General Hospital, Santa Fe, N. M.
[3] August, 1943 to August, 1946.

Distribution of lesions: The majority of the lesions were in the right and left lower lobes. The right lower lobe was more frequently the site of initial involvement than the left lower lobe. In 49 of the 90 cases in the group, the disease



FIGS. 1–3

FIG. 1. Localization of lesion by lobe in 2,777 cases of tuberculosis.

FIG. 2. Ninety cases of lower lobe tuberculosis. Distribution of lesions between right middle and both lower lobes.

FIG. 3. Ninety cases of lower lobe tuberculosis. Localization of lesion within the lobe.

first appeared in the right lower lobe and in 37 cases the first lesion was in the left lower lobe. The initial lesion occurred in the right middle lobe in only 4 instances (figure 2).

Location of disease process within the individual lobe: This particular question

was studied to determine whether there was any selective distribution in lower lobe disease. Analysis as to whether the extent of the involvement was predominantly in the upper half or the lower half was carried out. Eighty-nine of the 90 case records presented sufficient information to permit this classification. The upper half of the lobe was involved twice as frequently as the lower half. Forty-six had upper lobe distribution. In contrast, 23 demonstrated lower half involvement. Of the remainder, 20 showed no specific selection but involved the entire lobe (figure 3).

Radiopathological description: In 62 (72.9 per cent) of the reinfection cases, cavitary disease was evident on the first chest film obtained after hospitalization. In the remainder (27.1 per cent) only infiltrations were present. There was no unusual incidence of cavitation in any particular lobe.

Qualitatively, there was nothing characteristic of the infiltrations in the lower lobes. As might be expected, the majority (53) were interpreted as "mixed" disease. Twenty were considered to be "productive" and 5 "exudative". The only "calcific" lesions were the 5 primary complexes. The remaining records (7) were not classified in this respect.

Race and age: As compared to the overall pulmonary tuberculosis census of the hospital, there was no difference in the incidence of lower lobe disease with regard to race or age. Similarly, there was no specific lobe distribution. The series included 18 Negroes, 2 Eskimos, and one Indian. The age range was 18 to 46 years; the average was 25 years.

RESULTS OF TREATMENT

The results of treatment were studied with reference to the factors of incidence and also the types of treatment used.

It was appreciated that the period of observation was short. The time ranged from one month to two years, the average being nine months to one year. As final military disposition divided this group into numberous destinations[4], it was considered unlikely that final observation as a unit would ever be possible. Therefore, a report of the results observed in this relatively brief period of observation seemed justifiable.

Results with Reference to Factors of Incidence

Relation to involved lobe: The lobe involved offered no prognostic clue. In 32 (65.1 per cent) of the 49 cases with right lower lobe involvement, the disease improved. There was no significant difference with the left lower lobe lesions which showed improvement in 26 of the 37 cases.

Location of disease process within the individual lobe: The particular location of the lesion within the lobe was likewise of no prognostic value. Of 62 lesions that showed improvement, 32 (51.6 per cent) were located in the upper half of the involved lobe. The other 30 were equally distributed between those cases which

[4] Seventy-six of these patients were transferred to other sanitoriums to continue treatment; 5 were returned to duty with healed primary disease, and 9 separated from military service with inactive disease.

involved the lower half of the lobe and those cases with involvement throughout the lobe. Likewise, there was no apparent relationship between lack of improvement and location of the lesion within the lobe. In thirteen (61.9 per cent) of the 21 patients whose disease failed to improve, the predominant involvement was in the upper half of the lobe.

Race: The tuberculosis had an unfavorable course in the 2 Eskimos in this group of patients. One had moderately advanced disease; the other far advanced. Both were treated only with bed rest. With this possible exception there was no correlation between the results of treatment and racial types.

Age: No significant correlation was noted between the clinical course of the lower lobe disease and the age of the patient. The average age for those who improved was 24.6 years, with a range from 19 to 39 years. The average age for those with an unfavorable course was 25.8 years, with a range from 18 to 46 years.

Results Analyzed According to Types of Treatment

Method of Study: Three major groups could be defined, determined by the types of therapy in frequent use: (*1*) bed rest alone; (*2*) pneumothorax; and (*3*) lobectomy.

Several other procedures, pneumoperitoneum, phrenic nerve interruption, and the combination of these two, formed a miscellaneous group.

Each of these groups was divided into two categories: those cases showing improvement; and those cases showing no improvement (including progressive disease).

The criteria for improvement included one or more of the following points:

1. Clinical improvement, as judged by gain in weight, absence of fever, and decrease to absence of sputum and cough.
2. Roentgenological improvement as evidenced by study of serial roentgenograms.
3. Disappearance of acid-fast bacilli from sputum examined after concentrations.

Individual reference was noted in the response of cavitary disease to treatment. The group was studied with regard to: size of cavity (diameter) prior to treatment; degree of cavity closure; and reversal of infectiousness.

Comparison of results obtained by various methods of treatment: Improvement in the course of the disease occurred in: 41 (71.9 per cent) of the 57 patients treated with bed rest alone; 13 (68.4 per cent) of the 19 treated with pneumothorax; and 6 (85.7 per cent) of the 7 patients in whom lobectomy was performed (figure 4).

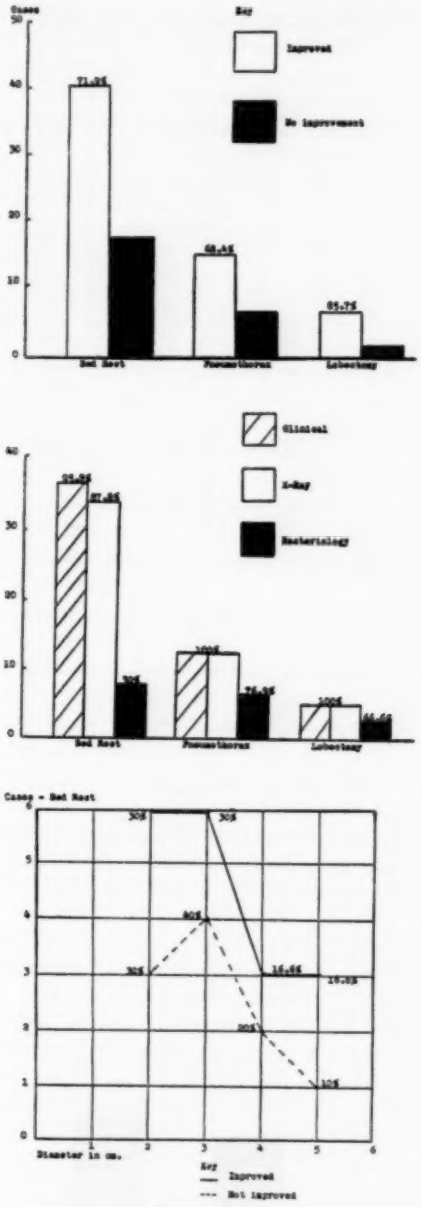
On study of the results of treatment with reference to the individual points mentioned under criteria for improvement, it was the writers' impression that pneumothorax and lobectomy effected a greater degree of improvement than bed rest alone. There was clinical as well as roentgenological improvement in the lesions of those 19 patients who showed improvement with pneumothorax or underwent lobectomy. Conversely, in only 36 (87.8 per cent) of the 41 cases in which improvement occurred on bed rest alone was there both clinical and roent-

---

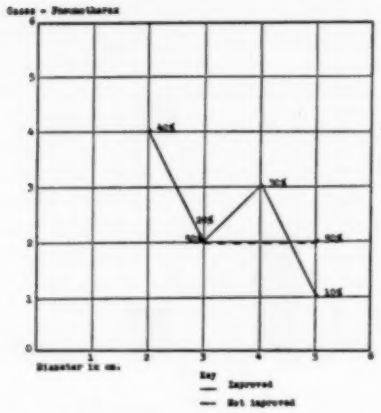
FIG. 4. Results of three forms of treatment of 90 cases of lower lobe tuberculosis.

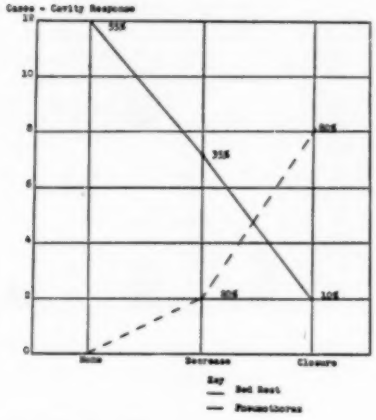
FIG. 5. Clinical, roentgenologic and bacteriologic results of three forms of treatment of lower lobe tuberculosis.

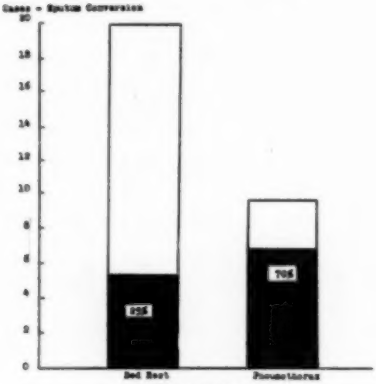
FIG. 6. Lower lobe tuberculosis. Change in cavity diameter under treatment by bed rest.


Cases
Key
Improved
No improvement
71.4%
68.4%
85.7%
Bed Rest
Pneumothorax
Lobectomy
Clinical
X-Ray
Bacteriology
95.8%
97.4%
30%
100%
76.9%
100%
66.6%
Bed Rest
Pneumothorax
Lobectomy
Cases - Bed Rest
30%
30%
40%
30%
16.6%
16.6%
20%
10%
Diameter in cm.
Key
Improved
Not improved

FIGS. 4-6


Cases - Pneumothorax
Diameter in cm.
Key
Improved
Not improved


Cases - Cavity Response
Holes
Decrease
Closure
Key
Bed Rest
Pneumothorax


Cases - Sputum Conversion
95%
70%
Bed Rest
Pneumothorax

FIGS. 7–9

genological improvement. Of the remainder of this "bed rest" group, only clinical improvement was observed in 3 cases and only roentgenological evidence of improvement in two others.

In all of the cases in the pneumothorax and lobectomy groups the sputum was positive for tubercle bacilli prior to treatment. Tubercle bacilli disappeared from the sputum in 10 of the 13 patients whose disease improved with pneumothorax and in 4 of the 6 with improvement after lobectomy. In the "bed rest" group, 30 of the 41 patients whose disease improved had tubercle bacilli in the sputum on the initial examination, and in 9 of the 30 the sputum subsequently was negative for tubercle bacilli (figure 5).

Response of Cavitary Disease: There were 48 patients with cavitary disease. The diameter of the cavity was recorded in 42 cases. It ranged from 2 to 5 cm. The average was 3.1 cm.

There was no correlation between size of cavity and therapeutic response. Twenty-eight cases were treated by only bed rest; 18 improved, 10 did not improve. Twelve of those that improved and 7 of the unimproved cases had an average range in cavity diameter of 2 to 3 cm. The remainder of each group had a range of 4 to 5 cm. (figure 6). The cavity diameter in 6 of the 10 cases that improved with pneumothorax ranged between 2 to 3 cm.; in the remainder, the cavities were 4 to 5 cm. in diameter. In 2 of the 4 patients whose disease failed to improve, cavities 3 cm. in diameter were present; and in the other 2, the cavities were 5 cm. in diameter (figure 7).

Pneumothorax appeared to be more effective in obtaining cavity closure and sputum "conversion" than bed rest alone. Cavity closure occurred in only 2 of the 20 cases of cavitary disease in which improvement occurred on bed rest, while this objective was attained in 8 of the 10 cases that received pneumothorax. Sputum "conversion" occurred in 5 cases treated only with bed rest and in 7 of the pneumothorax cases (figures 8 and 9).

Pneumoperitoneum and phrenic interruption: Pneumoperitoneum and phrenic interruption were used in only 5 cases. Possibly one of the reasons why these procedures were not used more frequently was that the consensus of opinion at the weekly chest conferences, where therapy for the cases was discussed and decided, did not favor these procedures with the same enthusiasm as might be held by others.

Three patients with cavitary disease were selected for pneumoperitoneum. In one, diaphragmatic paralysis was present and, in the others, no phrenic interruption was performed. The cavity diameter ranged from 2.5 to 4 cm. All cavities were located in the upper half of the left lower lobe. None of the cases demonstrated any improvement after four to six months of observation under such therapy.

Another 2 received phrenic interruption alone. Both had cavitary disease of

FIG. 7. Lower lobe tuberculosis. Comparison of change in cavity diameter observed after treatment with bed rest or pneumothorax.

FIG. 8. Lower lobe tuberculosis. Comparison of change in cavity diameter observed after treatment with bed rest or pneumothorax.

FIG. 9. Bacteriologic results of treatment with bed rest or pneumothroax.

the upper half of the left lower lobe, and the cavities ranged from 2 to 3 cm. in diameter. No change in the status of the disease was noted after approximately four months of therapy.

Discussion

In this study the general incidence (3.1 per cent) of reinfection type of lower lobe disease compared favorably with other reported studies. According to Romendick, Friedman and Schwartz (3), the incidence may vary from 0.003 per cent to 18.3 per cent. In their own series of 2,354 cases, 63 (2.7 per cent) had lower lobe disease. Lathrop and Lyman (4) found an incidence of 3.1 per cent, 83 cases in a series of 2,809 cases. On the other hand, Reisner (5) reported only 0.8 per cent lower lobe involvement, 34 cases in a series of 4,494 cases.

Incidence of the lower lobe most frequently involved in the present series compared favorably with the previous reports. In 54.4 per cent of the present series, the involvement was in the right lower lobe. Romendick *et al.* (3) reported 58.7 per cent with involvement of the right lower lobe and Hawkins and Thomas (6), and Sokoloff (7) also found the right lower lobe more frequently involved.

The greater frequency with which tuberculosis occurred in the upper half of the lower lobe has also been noted in other studies. Ossen (8) observed that lesions occurred in the apex of the lower lobe more frequently than in the basilar portions. Sokoloff (7) reported 10 lesions located close to the root of the lung or in the upper third of the lower lobe. One of the factors in the pathogenesis of this localization might be found in the work of Sweany, Cook and Kegerreis (9). They studied the position of primary cavitation in pulmonary tuberculosis and found that the apices of the lower lobes frequently contained the oldest lesion in the anatomical specimen. Viswanathan (10) discussed the physiologic interpretation of the apical localization and believes it represents a predisposition to disease because of inadequate ventilation and poor circulation in the upper portion of the lower lobe.

The tendency for lower lobe tuberculosis to become cavitary disease (72.9 per cent of the cases in this series) is emphasized in other reports in the literature. Reisner (5) commented that the majority of his cases had cavernous disease. Sokoloff (7) observed in his series that there was a tendency for rapid development of cavernous disease.

The age range, 18 to 46 years, with an average of 25 years, followed closely the ranges stated by other writers on this subject. Romendick *et al.* (3) reported that 91.5 per cent of their cases were under 40 years of age. In Reisner's group two-thirds were between the ages of 17 and 30 years, and Viswanathan (10) reported a range of 17 to 42 years. Like this study, Romendick *et al.* (3), Reisner (5), and Weidman and Campbell (11) could find no definite relationship between the race of the patient and the incidence of lower lobe disease. Though this series included only males, a greater incidence in females is reported in the literature (5, 10, 11).

Weidman and Campbell (11) reported 6 cases treated by only bed rest. Four obtained a satisfactory result; 2 had cavitary involvement. Although improve-

ment was noted in 71.9 per cent of the cases in the present study treated with bed rest, in only 15 of 57 cases was the response satisfactory with clinical and roentgenological improvement and sputum "conversion." Only 2 of this group of 15 had cavitary disease.

There has been considerable difference of opinion as to the choice of collapse procedure. Pohl (12) mentioned pneumothorax as the preferred treatment. In contrast, Hawkins and Thomas (6) reported pneumothorax ineffective in 11 of 15 cases of lower lobe tuberculosis. Weidman and Campbell (11) stated that 6 of 15 cases treated by pneumothorax obtained a satisfactory result. Reisner (5) reported pneumothorax effective in 58 per cent of his cases, 11 in a series of 19. Of the 19 cases in the present study, 13 showed improvement, and 10 had a satisfactory result (clinical and roentgenological improvement with sputum "conversion").

Phrenic interruption and pneumoperitoneum were ineffective in the 5 cases in which it was used. Hawkins and Thomas (6) contrasted their poor results with pneumothorax with the response, including cavity closure, noted in 10 cases treated by phrenic interruption and pneumoperitoneum. They emphasized the ineffectiveness of phrenic interruption unless supplemented by pneumoperitoneum. The results of Weidman and Campbell (11) and Reisner (5) supported this opinion. The former reported that all 4 cases in their series treated by phrenic paralysis had poor results. Reisner (5) found phrenic interruption effective in only one of 8 cases so treated.

Carr and Harter (13) recommended pulmonary resection for that group of patients without reasonable hope of cure by collapse therapy. In this group they also included lower lobe disease not amenable to collapse treatment. In a series of 60 resections by Overholt and Wilson (14), 10 were lower lobe disease. All were improved or became clinically well after the procedure although in 5 the sputum was positive for tubercle bacilli when the cases were reported. In Bailey's (15) series of 80 resections, 4 were for lower lobe disease; 2 were improved. In the lobectomy group discussed in this paper there were 7 patients. Six improved or became clinically well; 2 of these patients had persistently "positive" sputum postoperatively.

In addition to resection, the use of thoracoplasty for lower lobe disease has been discussed in the literature (3, 7, 16). Freelander (16) suggested its use in persistent basal cavities. Romendick *et al.* (3) reported 4 cases by thoracoplasty; only one became arrested.

Summary

Observations on lower lobe involvement in pulmonary tuberculosis are reported. The source of the material consisted of 2,784 successive admissions for pulmonary tuberculosis at an Army General Hospital:

1. Lower lobe disease was found in 85 cases or 3.1 per cent of the group.
2. Disease of the right lower lobe was slightly more frequent than of the left lower lobe.
3. The upper half of the involved lobe became the initial focus of the disease more often than the basilar portion.

4. There was a tendency for rapid development of cavitary disease.

5. Pneumothorax and lobectomy were effective therapeutic adjuncts in this group of cases.

Sumario

Observaciones Relativas a la Enfermedad del Lóbulo Inferior en la Tuberculosis Pulmonar

Estas observaciones referentes a la invasión del lóbulo inferior en la tuberculosis pulmonar proceden de 2,784 ingresos sucesivos por tuberculosis pulmonar en un Hospital General del Ejército E. U. A.:

1. Descubrióse afección de los lóbulos inferiores en 85 casos, o sea 3.1 por ciento del grupo.

2. La enfermedad del lóbulo inferior derecho fué ligeramente más frecuente que la del izquierdo.

3. La mitad superior del lóbulo afectado se convirtió en foco inicial de la enfermedad más a menudo que la porción basal.

4. Observóse tendencia a rápida formación de cavernas.

5. En este grupo el neumotórax y la lobectomía mostraron su efectividad como coadyuvantes terapéuticos.

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INSULIN IN THE TREATMENT OF TUBERCULOUS PATIENTS WITH ANOREXIA—A MODIFIED TECHNIQUE[1, 2]

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INTRODUCTION

The use of insulin for the treatment of anorexia and undernutrition in tuberculous patients was widely discussed in both the European and the American medical literature from 1923 on through the late thirties (1, 2, 3, 4, 5, 6, 7, 8). Although the majority of authors reported favorable results, others claimed that it produced no constant or lasting gain in weight and did not alter the course of the disease. Furthermore, they warned of possible deleterious effects of hypoglycemia on the tuberculous process, particularly in cases of exudative disease. The proponents of insulin therapy failed apparently to present a sufficiently convincing case and, instead of becoming widely accepted, the method gradually fell into disuse.

During World War II several thousand cases of anxiety neurosis were treated with sub-shock doses of insulin (9, 10) and attention was again called to its efficacy in improving appetite and nutrition. Insulin was also found to have a noticeable sedative action: "Symptoms of acute anxiety, tremor, perspiration, restlessness, and insomnia will tend to improve toward the end of the first week" (Kelly and Thompson (11)).

The idea of using insulin in sub-shock doses to promote weight gain in anorexic tuberculous patients was suggested to the present writers by Dr. Irving Pine of Asheville, North Carolina, who had noted the enormous weight gain of psychiatric patients receiving large doses of insulin.

It is not difficult to understand why many a patient in a tuberculosis hospital develops anxiety. He is aware of the fact that he has a serious chronic disease and he may actually have seen or known others who have died of this disease. He worries about his family and how they will manage financially during his prolonged illness. He wonders whether he will be able to return to his job and his friends after recovery, or whether he will be regarded as an outcast. The mention of "thoracoplasty" produces fear of mutilation and death. Pneumothorax and pneumoperitoneum mean the dreaded needle and the complications of which he has heard the other patients talk. A previous hemoptysis or a spontaneous pneumothorax may further have implanted the overpowering fear of suffocation. This continued emotional stress helps to explain why some tuberculous patients will continue to eat poorly and lose weight even after all fever has gone and the

[1] From the Department of Medicine and Surgery, Veterans Administration, Oteen, North Carolina.

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pulmonary disease shows clinical and radiologic improvement. The habit of anorexia has been established and must be broken. Insulin treatment is of value in "breaking down the vicious cycle of anxiety and loss of weight" (9).

OBSERVATIONS

Technique

Until now the only way in which insulin has been used to produce weight gain has been in small doses given at fairly short intervals before feedings. The technique used in this study consisted of giving regular insulin subcutaneously in single daily doses, increased daily by 5 or 10 units from a starting level of 20 units to as high as 50 or 60 units. This final figure has been found to be effective in most patients and is continued for a total period of one to two months. The insulin is given to the fasting patient at 6:00 a.m. and no food is taken until breakfast is served at 8:00 a.m., by which time a voracious appetite has been aroused and occasionally a feeling of faintness. A normal hospital meal schedule is resumed for the noon meal at 11:30 a.m. and supper at 5:00 p.m. It has been found that the increased appetite created for breakfast survives to a lesser degree for the other meals.

Composition of Clinical Series

Sixteen white male veterans at Oteen Veterans Administration Hospital, ranging in age from 18 to 35 years, were treated as described. These men had been hospitalized at Oteen for periods of six months to four years and all of them had received one or more of the following forms of collapse therapy: pneumothorax, pneumoperitoneum, phrenic crush, thoracoplasty, and extrapleural lucite pack. All were afebrile, showed stationary or slowly regressing disease, and complained of poor appetite for the past two to six months.

RESULTS

Of the 16 patients treated with insulin in sub-shock doses, all registered some gain in weight. Nine gained between ten and twenty pounds, and 7 gained from two to nine pounds, in a period of four weeks. Follow-up observations for six to eight weeks after insulin was discontinued show that one-half of the patients lost approximately 25 per cent of the weight they had gained. Five patients either maintained their gain in weight or added further weight. Three patients left the hospital "AWOL" and their progress could not be followed. All of the 13 patients who remained in the hospital agreed that their appetite now was definitely better than it had been prior to receiving insulin. In none of the patients treated was any definite exacerbation of the tuberculous process noted either during or six to eight weeks after the course of insulin therapy.

The following case reports are illustrative of the clinical course observed during insulin therapy.

CASE REPORTS

Case 1: M. D., a 35 year old man with known pulmonary tuberculosis for one year, complicated by left pleural effusion, was treated with bed rest and pneumoperitoneum. His appe-

tite was poor, particularly at breakfast, at which time he would usually take only a cup of coffee and a small amount of milk. During rest hour he would fidget about in bed and his legs would jerk aimlessly. He complained of frequent heartburn. He was started on 20 units of regular insulin which was increased by 10 units daily up to 50 units once a day. Beginning on the second day of treatment there was a noticeable increase in appetite, most marked at breakfast but to a lesser extent also at the noon and evening meals. For breakfast the patient was now able to eat four eggs, five or six slices of toast, a glass of fruit juice, a bowl of cereal with a glass of milk and a cup of coffee. He also noted a definite sedative action. He slept and rested better and his feet stopped "jumping." He gained 8 pounds in a little less than a month.

Case 2: B. F. was a 24 year old man with known tuberculosis for over three years, complicated by tuberculous laryngitis which was healed at the time of insulin therapy. He had fibroexudative disease scattered throughout both lungs, which was satisfactorily treated by a combination of left pneumothorax and pneumoperitoneum. For about a year before treatment his weight remained constant at about 20 pounds below his normal level. In late November 1947, he began to complain of early morning nausea which grew gradually worse until no appreciable amount of food was tolerated by mouth. All methods, including intravenous feeding, were tried without breaking through the vicious cycle. In January 1948, insulin was tried; the response was rapid and dramatic. The acute episode was aborted and a ravenous appetite resulted in a gain in weight from 144 to 164 pounds in a little less than two months.

Case 3: W. R. was an 18 year old adolescent who had had tuberculosis for one year. Sputum was positive for tubercle bacilli at the onset, but converted with a right pneumothorax after an open pneumonolysis had been performed. This patient presented a great behavior problem, having had no adequate discipline and guidance. He came from a small town in Kentucky where almost the only type of occupation available was work in the mines or the sawmills, and he felt that his pulmonary disease would prevent hin from handling these jobs. His horizon extended no further. He was extremely tense, nervous, anxious, and insecure. His appetite was very poor, with no response to any medical measures. Insulin treatment resulted in the creation of an enormous appetite and a gain of 17 pounds in weight in one month.

Case 4: F. H. was a 22 year old man with known tuberculosis for seven months, which had been adequately treated with left phrenic crush and pneumoperitoneum, with consequent reversal of infectiousness. The patient claimed that he had been a "good eater" before entering the hospital. For the previous six months, however, he had suffered from "nervous stomach", poor appetite, and occasional diarrhea. Medical measures to stimulate appetite were of no avail. Insulin resulted in a prompt and marked improvement in appetite and a gain in weight of from 117 to 127½ pounds in one and one-half months.

Case 5: W. A. was a 24 year old veteran with known cavitary tuberculosis for four years, which had been treated first with left phrenic crush and pneumoperitoneum and then, in 1947, with a left extrapleural lucite pack which failed to close the cavity. Less than a year later the lucite balls were removed and a thoracoplasty was performed. This man had been extremely anxious over the long course of his disease without much improvement, and was very irritable and fretful. Under insulin therapy his mental outlook became dramatically better, he was cheerful and cooperative, and he gained 13½ pounds in a little less than one month's time.

Case 6: J. P. was a 31 year old veteran with known far advanced pulmonary tuberculosis for over five years, who had been treated successively with right pneumothorax, right thoracoplasty, left pneumothorax, and pneumoperitoneum. The patient's chronic illness had re-

sulted in a weight loss that remained persistently at about 60 pounds below his normal level. For ten days he was given sterile water injections but was otherwise treated just like the other insulin patients. This resulted in no subjective improvement and he lost one pound. On starting insulin in the second week the patient noted at once the effect on his appetite and he gained 5 pounds in two weeks.

TABLE 1

Weight Data on Patients Treated with Insulin

	I	II	III	IV	V	VI	VII
	Normal weight	Weight on admission	Lowest weight recorded during illness	Weight before treatment	Weight after one month of treatment	Net gain	Weight 6 to 8 weeks after end of treatment
	pounds	*pounds*	*pounds*	*pounds*	*pounds*	*pounds*	*pounds*
M. D. (1)	150	153	139	143	151½	8½	153
B. F. (2)	165	155	144	144	164	20	160
W. R. (3)	120	112	109	109	126	17	AWOL
F. H. (4)	135	120	117	117	127½	10½	126½
W. A. A. (5)	145	135	87	101	114½	13½	114
J. P. (6)	165	165	104	117½	123	5½	124
J. E. M. (7)	140	135	119	127	138	11	134½
L. B. (8)	160	144	118	124	140	16	140
M. C. (9)	145	139	132	135	140	5	AWOL
R. C. (10)	143	129	119	133½	145	11½	AWOL
R. H. (11)	155	98	98	122	130	8	128
J. S. (12)	157	147	140	146	148	2	148
J. M. (13)	132	117	110	110	120	10	117
H. G. (14)	160	113	112½	112½	126	13½	128
F. M. (15)	165	139	137	139	142½	3½	141½
P. D. (16)	145	120	119¼	119¼	126	6¼	122

Case 7: J. M., a 37 year old man with known pulmonary tuberculosis for five years, was treated successively with right pneumothorax, left pneumothorax, right phrenic crush, and pneumoperitoneum, and finally with right thoracoplasty and streptomycin. His appetite had fallen off just before the chest surgery and his weight had dropped from 140 to 127 pounds. He admitted that prior to the thoracoplasty he had worried a great deal about the possible physical deformity resulting from the operation. Furthermore, he had sustained some disturbance of vestibular function as a result of streptomycin treatment and was much concerned about his persistent dizziness. On insulin therapy this man gained 11 pounds in one month.

The pertinent weight details on all 16 patients treated with insulin may be seen in table 1.

DISCUSSION

The method of use of insulin in these underweight and anorexic tuberculous patients differs in two major respects from that heretofore employed. In the past it has been the uniform practice to give small doses (3 to 20 units) from twenty to forty-five minutes before meals, two or three times a day. In the present study single, daily, relatively large (50 to 60 units) "sub-shock" doses

have been given on an empty stomach in the early morning with breakfast served about two hours later.

This higher dose and the delay between injection and feeding usually allow the blood sugar to fall to about 75 per cent of its normal value. It is well established that a 25 per cent fall in blood sugar level will induce marked gastric secretion and motility (12, 13). It is interesting that physiologists have long known that these effects require at least seventy-five to ninety minutes to reach their peak, yet most clinical investigators in reporting the use of insulin for appetite stimulation have allowed only twenty to forty-five minutes between injection and feeding, which has not permitted maximum benefit. In the present study, the patients' appetite and hunger increased to a peak at about two hours after injection, succeeded by faintness if food was withheld. By serving food when the subjective desire for it was greatest, a very satisfactory increase in food intake was achieved.

In addition to stimulating hunger through increased gastric secretions and contractions, insulin in sub-shock doses may also contribute toward weight gain by its sedative effect on the patient. Most of the subjects noted a definite decrease in their restless fidgeting in bed and useless activity on the ward. Some were able to reduce or dispense with sedative drugs which they had been taking rather regularly. A few showed marked improvement in general outlook, with disappearance of many vague hypochrondriacal complaints. In this connection, however, it is necessary to emphasize the fact that, in the presence of well-established personality disorders, no fundamental change can be expected from sub-shock doses of insulin (14, 11).

The duration of the course of treatment with insulin was arbitrary. The patients were treated for one to two months, but courses lasting several months or repeated courses of one or two months with a month off in between have been used with the old technique (6). This seems to be a matter of individual adjustment. There is little to be gained from continuing the course after the patient has reached his previous normal level of weight or when he has become stabilized and ceases to gain further. If pushed up beyond his normal level, the absolute increment is apt to be lost as soon as treatment is terminated.

In any regimen of treatment, the role of suggestion is not to be underestimated. It has indeed been claimed (15) that insulin could not do more for anorexia and undernutrition than would a good diet and ample encouragement. In the cases here described, however, the diet was constant before, during, and after treatment; and suggestion, as well as miscellaneous medical measures (vitamins, "tonics," et cetera), had been tried without success. We feel that the insulin regimen abruptly breaks through a habit pattern of worry and chronic poor appetite, resulting in immediate weight gain (14, 16). The immense psychic boost of knowing that one is gaining weight and presumably getting generally better plays a considerable part, once the treatment is well under way, in perpetuating the weight gain, maintaining better appetite after treatment, and improving the whole mental outlook. In one case cited, suggestion combined with sterile water injections were used for ten days with no improvement (the weight dropped one pound); but when insulin was substituted for sterile water (unbeknown to the patient) an increase in appetite was noted at once with resultant gain in weight.

It has been suggested at various times that the weight gain of insulin-treated patients consists largely of water retention, and as such is of no lasting value. Careful studies of water balance (17), however, show that this is not the case.

Some writers (18, 2) have voiced warnings about possible deleterious effects of insulin in nondiabetics. The suggestion that it might induce disturbance of normal endocrine functions through suppression of islet cell activity has never been substantiated, and Allen (19) states categorically that he has heard of no instance of development of diabetes or of any other endocrine disorder. In the past, insulin has been given over extensive periods for many months, either continuously or in repeated courses, with no ill effects observed (20, 21). In a disease like tuberculosis, the principal deterrent to the use of insulin has been the fear that hypoglycemia may initiate exacerbations of exudative lesions. The mechanism of such exacerbation has never been clearly described and the feeling seems to be based on a few isolated reports of spread or hemorrhage while under insulin therapy. Closer scrutiny reveals that these were usually cases of active exudative or cavitary disease, in a certain number of which spreads and hemoptyses may be expected to occur with any type of treatment. No study has ever proved that the incidence of such complications was any higher in the insulin-treated series than in a comparable nontreated group. Nevertheless, however dubious the danger may be, it is one that cannot be absolutely excluded. Therefore, the patients for this study were chosen, not as a cross-section of tuberculosis sufferers, but as those most likely to be benefited, omitting all who might conceivably be adversely affected by insulin. The group chosen consisted of fairly young men who were afebrile, in whom tuberculosis was either stationary or gradually improving, who were anorexic and considerably below their normal weight, and who had not shown any tendency to gain weight under conservative management. Those excluded were: patients with active exudative pulmonary disease, patients with definite tuberculous enteritis, patients over 45, those with organic heart disease, and those who displayed sensitivity to insulin. All patients treated were under close supervision, and adequate materials were kept at hand for prompt oral or intravenous administration of glucose in case of insulin shock. With the doses used, no instance of insulin shock has been encountered in the present group of patients.

SUMMARY AND CONCLUSIONS

1. The use of regular insulin in sub-shock doses to stimulate appetite and weight gain in anorexic tuberculous patients has been discussed.

2. The element of anxiety is an important factor in the mechanism of anorexia in these cases.

3. Sixteen patients were treated for one month with single daily doses of 50 units of insulin. Weight gains were recorded in all patients, and 9 gained between 10 and 20 pounds.

4. It is believed that in properly selected cases of quiescent tuberculosis, where anorexia and failure to gain weight are a prominent part of the clinical picture, insulin in sub-shock doses is a valuable adjunct in treatment, and is without any appreciable danger.

SUMARIO Y CONCLUSIONES

La Insulina en el Tratamiento de los Tuberculosos Anoréxicos—Técnica Modificada

1. Discútese el empleo de la insulina corriente a dosis sub-choque para excitar el apetito y aumentar el peso en los tuberculosos con anorexia.

2. El elemento de ansiedad es un importante factor en el mecanismo de la anorexia en estos casos.

3. Dieciséis enfermos fueron tratados por un mes con dosis diarias únicas de 50 unidades de insulina, notándose en todos aumento de peso, que en nueve representó de unos 4.5 a 9 kg.

4. Parece que, en casos debidamente seleccionados de tuberculosis quiescente, en los que la anorexia y la falta de aumento de peso constituyen parte destacada del cuadro clínico, la insulina a dosis de sub-choque es un valioso coadyuvante terapéutico sin que entrañe riesgo apreciable.

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TUBERCULOSIS OF THE TRACHEA AND MAJOR BRONCHI[1]

Results of Treatment with Streptomycin

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(Received for publication November 16, 1948)

INTRODUCTION

Tracheobronchial tuberculosis has received much attention in recent years. It is recognized as a frequent complication of pulmonary tuberculosis and is a serious problem which significantly alters the prognosis of the pulmonary disease. Treatment frequently must be modified when a tracheal or bronchial lesion is demonstrated.

Bronchoscopic examination is essential to the diagnosis of tracheobronchial tuberculosis and ideally should be performed on all patients admitted to sanatoriums because of tuberculosis. Certainly it is indicated before any form of collapse therapy is instituted and whenever there are symptoms of bronchial obstruction, such as wheezing respiration, bouts of fever, or persistent cough with retention of secretions. Tuberculous tracheobronchitis should be suspected when the symptoms are out of proportion to those which are likely to be caused by the findings on the roentgenogram of the chest, when the roentgenograms reveal evidence of atelectasis, or when the sputum remains positive for tubercle bacilli without obvious reason.

Preliminary reports of the treatment of tuberculous laryngitis and tuberculous ulceration of the trachea and major bronchi with streptomycin have been most encouraging (1, 2). Ulcers of the larynx and tracheobronchial tree were reported to have healed with amazing rapidity in the few cases in which such treatment was carried out. It was feared that results would be less dramatic when a larger series of patients were treated with streptomycin. The experience of the Veterans Administration (3), however, has amply confirmed the earlier impression that ulceration of the larynx, trachea, and major bronchi responds most satisfactorily to treatment with streptomycin. The report from the Veterans Administration stated that the ulcers of all of the 81 patients treated had healed. Brewer and Bogen likewise have reported similar results in 44 cases (4).

The question of the permanence of cure in cases of ulceration of the tracheobronchial tree is a matter of great importance. The Veterans Administration reported only one recurrence in the series of 81 cases. Brewer and Bogen (4) recorded recurrence in 3 cases in a relatively brief period of follow-up. It is the purpose of this report to describe the results in 3 of the earliest cases of tracheal and bronchial ulceration in which treatment with streptomycin was employed. These patients have remained under close observation for from one and one-half to three years since treatment was completed.

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REPORT OF CASES

Case 1: A student nurse, aged 21 years, registered at the Mayo Clinic for routine annual physical examination on April 8, 1943. Routine roentgenograms of the chest revealed a minimal tuberculous lesion in the upper part of the right lung (figure 1a). Roentgenograms in 1940 and 1942 had revealed no abnormality. The patient was sent to Mineral Springs Sanatorium in May 1943. Tubercle bacilli were demonstrated by inoculation of guinea pigs with material obtained from the stomach during fasting. Treatment consisting of rest in bed was carried out and the appearance of the lesion improved progressively. The patient was dismissed from the sanatorium on June 5, 1944. Reactivation of the lesion was noted on a roentgenogram made in December 1944, and she was readmitted to the sanatorium. At this time moderately advanced tuberculosis of the upper part of the right lung and a small minimal lesion on the left were present. Pneumothorax was instituted on the right side. After collapse, apparent atelectasis of the right upper lobe was noted (figure 1b). The patient complained of wheezing respiration, and bronchoscopic examination was carried out in March 1945. Ulceration was noted in the right main bronchus and on the right lateral aspect of the lower part of the trachea (figure 2). There was a definite stricture of the right main bronchus.

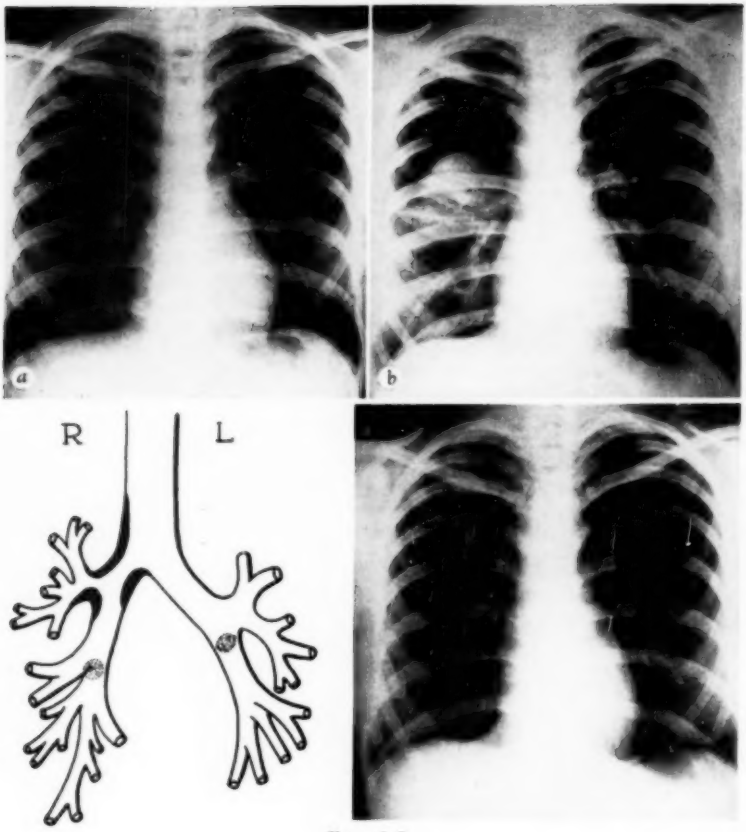
The patient returned to the clinic and was hospitalized in March 1945. Treatment with 0.5 Gm. of streptomycin aerosol daily and 1.2 Gm. of streptomycin by intramuscular injection daily was instituted. She remained under treatment for one month. Subsequent bronchoscopic examinations showed progressive improvement with healing of ulceration and inflammatory reaction within five weeks after therapy was begun. After her return to the sanatorium in April 1945, 2 Gm. of streptomycin were given intramuscularly each day for the next three months.

Bronchoscopic examination has been repeated at regular intervals in the past three years and there has been no return of ulceration. A stricture of the right main bronchus reduces its lumen to half normal size. The atelectasis of the right upper lobe disappeared, however, when the pneumothorax was discontinued (figure 3). The patient returned to the school of nursing in July 1947, and was working full time in July 1948, over three years since apparent healing of the bronchial tuberculosis. Repeated cultures of gastric contents obtained during fasting have been negative for tubercle bacilli.

Case 2: A woman, 37 years old, registered at the clinic on November 15, 1945. In the three years prior to coming to the clinic she had had several episodes of cough, wheezing, and difficult respiration. She had spent two years in a sanatorium because of tuberculosis. Although the roentgenogram revealed only a minimal lesion at the apex of the left lung (figure 4), her sputum was consistently positive for tubercle bacilli. Bronchoscopic examination performed at the sanatorium one year previous to her visit to the clinic had revealed a tuberculous lesion of the left main and left upper lobe bronchi. Treatment had consisted of local application of 30 per cent silver nitrate. Although subsequent examinations showed evidence of improvement, the sputum had remained positive for tubercle bacilli and symptoms of bronchial obstruction had recurred.

General examination revealed nothing of significance except for a slight wheezing respiration. Direct smears of the sputum revealed acid-fast bacilli. The sedimentation rate was 60 mm. in one hour (Westergren). At bronchoscopic examination, a nodular ulcerating lesion was found on the left lateral wall of the trachea just above the carina (figure 5). Considerable granulation tissue was removed.

The patient was admitted to the hospital where streptomycin therapy was administered for sixty-four days. One gram was given by intramuscular injection each day and 0.5 Gm. by nebulization. Bronchoscopic examinations were performed at intervals of two weeks. Improvement was rapid and progressive. After a month of treatment the tracheobronchial tree was practically normal except for slight distortion of the orifice of the left main bronchus. Repeated bronchoscopic examinations have been carried out ever since and her improvement has been maintained.



FIGS. 1-3

FIG. 1 (case 1). a. (Upper left) April 8, 1943, minimal active pulmonary tuberculosis at the level of the second interspace anteriorly in the right lung. b. (Upper right) February 9, 1945, right pneumothorax for reactivated pulmonary tuberculosis. Atelectasis of right upper lobe. Minimal tuberculous lesions at the level of the second interspace anteriorly in the left lung.

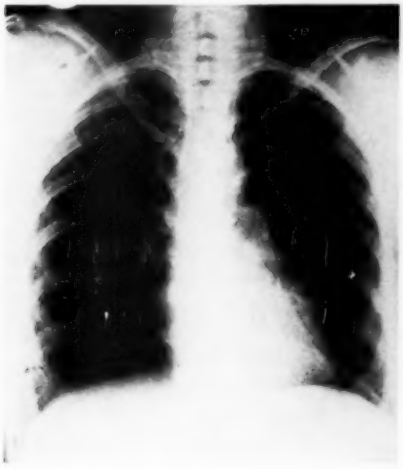
FIG. 2 (case 1). (Lower left) The bronchial tree. Areas marked with heavy black ink indicate distribution of tuberculous tracheobronchial lesions visualized at bronchoscopic examination.

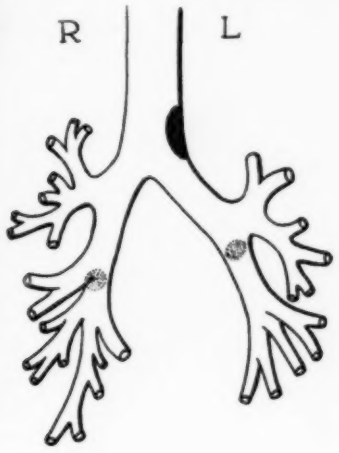
FIG. 3 (case 1). (Lower right) November 13, 1945. Five months after streptomycin therapy was discontinued. Chest negative except for residual linear fibrosis right upper lung field.

Daily examinations of sputum were carried out during treatment. Tubercle bacilli were found for two weeks after therapy was instituted but since then the sputum has been consistently negative. Guinea pigs inoculated with her fasting gastric content on several occasions since treatment have not developed tuberculosis. Sedimentation rate has

dropped to 18 mm. per hour. There has been no evidence of recurrence of tuberculosis up to the present time (July 1948), two years and seven months since the bronchial lesion appeared to have healed.

Case 3: A nurse, 46 years of age, registered at the clinic on September 10, 1946. Pulmonary tuberculosis and tuberculous laryngitis had developed in 1932. After several months of rest in bed and silence therapy, the tuberculosis was considered arrested and she returned to her work as a nurse. She remained well until May 1946, when a routine roentgenogram of the chest revealed reactivation of tuberculosis in the apex of the left lung. Rest in bed was advised. The sputum contained tubercle bacilli. Toward the end of May 1946, wheezing, dyspnea, and pain in the throat developed. Bronchoscopic examination revealed an ulcerative process in the lower part of the trachea and in the right main bronchus,





FIGS. 4-5

FIG. 4 (case 2). (Left) November 16, 1945, minimal tuberculous lesion in apex of left lung.

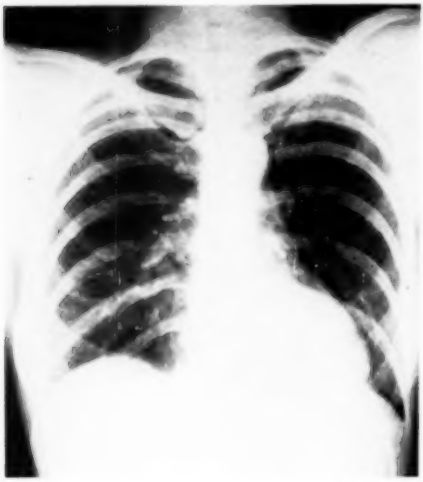
FIG. 5 (case 2). (Right) The bronchial tree. Area marked with heavy black ink indicates distribution of tuberculous tracheobronchial lesion visualized at bronchoscopic examination.

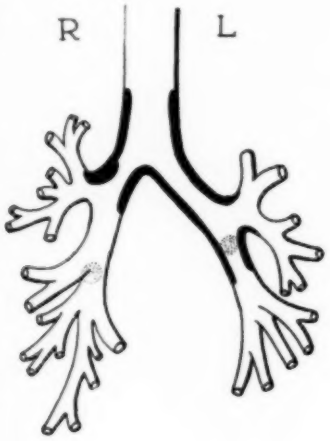
with considerable narrowing of the orifice of the right main bronchus. Subsequent progress was not favorable and because of fever, increasing dyspnea, and wheezing, she was sent to the clinic for treatment.

On arrival on September 10, 1946, she was hospitalized. Respirations were markedly obstructed and she was obviously ill. Acid-fast bacilli were demonstrated on direct smear of sputum. The sedimentation rate was 51 mm. in one hour (Westergren). Roentgenograms of the chest revealed a minimal degree of tuberculosis in the apex of the left lung and at the level of the left first interspace anteriorly (figure 6). On bronchoscopic examination an extensive ulcerating process was found in the lower part of the trachea. The ulceration extended into the left main, left upper, and left lower lobe bronchi (figure 7). The ulcers were covered with granulations and a tenacious gray mucus. There was an inflammatory stricture of the right main bronchus which reduced the orifice to a diameter of 5 mm. The mucosa near the carina was studded with nodules which grossly appeared to be tubercles.

Considerable granulation tissue and secretion were removed and raw bleeding surfaces were left. The patient was able to breathe much better at the conclusion of the examination.

Treatment with streptomycin was instituted on September 12, 1946, and continued for seventy-six days. Streptomycin, 1.5 Gm. in divided doses, was administered by intramuscular injection daily. One-half gram dissolved in 10 cc. of saline solution was nebulized and given each day by inhalation. Daily examinations of sputum were carried out. Nearly all specimens were positive for tubercle bacilli for the first month of treatment. After that it became difficult to demonstrate tubercle bacilli by concentration methods. In the last three weeks of her treatment at the clinic, a few bacilli were demonstrated microscopically on two occasions. The erythrocyte sedimentation rate dropped to 15 mm. in one hour.





FIGS. 6-7

FIG. 6 (case 3). (Left) October 5, 1946. Minimal tuberculosis in apex of left lung and anteriorly at level of the left first interspace.

FIG. 7 (case 3). (Right) The bronchial tree. Areas marked with heavy black ink indicate distribution of tuberculous tracheobronchial lesions visualized at bronchoscopic examination.

Rather severe symptoms of vestibular dysfunction developed after one month of treatment with streptomycin and it was necessary to discontinue treatment for one week. Parenteral therapy with 1.5 Gm. of streptomycin daily was then resumed but no further drug was given by inhalation.

Bronchoscopic examination, performed two weeks after streptomycin therapy was begun, revealed striking improvement. The ulcers had disappeared from the trachea and were less marked in the left main and lower lobe bronchi. Continued improvement was observed at subsequent bronchoscopic examinations and at the final examination on November 13, 1946, there was no residual ulceration and the mucosa was entirely clean. There was some contraction of the lower part of the trachea but the stenosis of the right main bronchus was less marked and the bronchoscope could be passed into the right lower lobe bronchus.

The patient returned to the sanatorium seventy-eight days after registering at the

clinic. Frequent reports concerning her progress had been obtained. Cultures of sputum and of gastric contents have been carried out repeatedly but none have been positive for tubercle bacilli. Bronchoscopic examinations performed at intervals have revealed that her improvement has been maintained, although she has some cicatricial contraction of the lower part of the trachea and the right main bronchus. There has been no evidence to suggest recurrence of tuberculosis during the one and one-half years since the bronchial lesions appeared to have healed.

COMMENT

The 3 cases reported demonstrate the prompt and apparently permanent healing of ulcerative tuberculosis of the trachea and bronchi which would never have been anticipated with any previous method of treatment. There has been no recurrence of disease in these patients during the interval of one and one-half to three years of observation since healing was achieved.[2] These cases were unique in that the parenchymal pulmonary tuberculosis in each was relatively minor as compared with the extensive and serious tracheobronchial lesions. It is obvious that antibacterial therapy could have no effect on fibrotic stenosis of the trachea and bronchi. Yet, when the ulcerating mucosal lesions healed in these cases, there was much less stenosis than was anticipated, and pulmonary resection which had been considered was unnecessary.

Equally dramatic results have been observed in 3 additional cases. Two of these patients had had thoracoplasty previously for extensive parenchymal disease but bronchial ulceration had persisted and probably had progressed. Prompt healing of the bronchial ulceration was observed after treatment with streptomycin but considerable fibrotic stenosis persisted. Cultures of the sputum and of gastric contents obtained during fasting were converted from "positive" to "negative" in both cases. The third patient responded favorably to treatment for tuberculous disease involving the mucosa of the left main bronchus with extensive ulceration and narrowing but eventually required left pneumonectomy.

The use of streptomycin has apparently reduced the danger of bronchoscopic manipulation and biopsy in the presence of tuberculosis. We no longer hesitate to remove granulation tissue from the trachea or bronchi, take specimens for biopsy, or dilate stenotic bronchi of tuberculous patients. In fact, the removal of obstructing tissue is often apparently essential to the complete treatment of such lesions.

The present trend in streptomycin therapy for tuberculosis is toward reduction in dosage. Our present practice in the treatment of tracheobronchial tuberculosis is to administer 1.0 Gm. of streptomycin daily in two equally divided doses at twelve hour intervals or at a single injection. Even smaller doses may prove to be adequate. It has been demonstrated clearly that the parenteral method of administration is far more effective than the aerosol method alone in the treatment of these lesions (3). There is some evidence to indicate that combined

[2] Since this paper was prepared for publication, the 3 patients whose cases are reported herein have been observed further. All of them have remained well and there has been no recurrence of the tracheobronchial lesions.

therapy may be more rapidly effective than injection therapy only. If aerosol therapy is to be used, it is recommended that 0.5 to 1.0 Gm. of streptomycin be given daily in a concentration of 100 mg. per cc. of physiologic salt solution.

The same toxic reactions which have been observed in most patients treated with streptomycin for any period of time were observed in this group of patients who had tracheobronchial lesions. Vestibular complications were frequent and were troublesome in some of the early cases but should be much less in future cases in which smaller doses of streptomycin will be used.

The excellent results obtained in cases of tracheobronchial tuberculosis have gone a long way toward establishing the efficacy of streptomycin in the treatment of tuberculosis. The introduction of streptomycin therapy has changed the attitude of the medical profession toward tuberculosis of the tracheobronchial tree. What was previously regarded as a most serious complication is now shown to be amenable to therapy. Early and accurate diagnosis of tracheobronchial tuberculosis becomes of great importance now that an effective therapeutic procedure is available.

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TOXICITY OF STREPTOMYCIN FOR THE AUDITORY AND VESTIBULAR MECHANISMS[1, 2, 3]

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When streptomycin is administered in high dosage, if poorly excreted, or if it is given over long periods of time, toxic reactions become an important consideration. Dysfunction of the vestibular mechanisms is the toxic reaction most frequently encountered. The patient complains of difficulty in walking under certain conditions, vertigo on sudden turning of the head, and trouble focusing his eyes, et cetera. Careful Hallpike (1) caloric measurements using 30° C. water for forty minutes or the Glorig mattress test (2) will often bring out dysfunctions which are not superficially apparent.

Reports from the Veterans Administration on 900 patients who received 1.8 to 2.0 Gm. of streptomycin per day state that 80 per cent of the patients had vertigo (3) by the fourth week of treatment. Although subjective vertigo usually disappeared, hypofunction or abolition of the vestibular response persisted. On one gram of streptomycin per day, vestibular disturbances developed in approximately 30 per cent of the patients by the sixtieth day of treatment.

At high dosage levels, auditory involvement is not infrequent and tends to follow vestibular loss if therapy is continued. On the other hand, it seems to improve if administration of the drug is discontinued before the damage is complete. We have seen 2 cases recover almost all of their hearing after having experienced quite a severe hearing loss. Incidentally, there is also some return of vestibular function in a few cases where the dosage was discontinued before total vestibular loss occurred. Auditory involvement in the lower dosage levels has very rarely been encountered.

Up to and including the present, most dosage reports and recommendations of dosages for therapy have been in grams of streptomycin per day. The same dosage is often given to both the small emaciated female weighing 38 Kg. and the average male of 70 Kg., or the large obese patient of 100 Kg. It will be shown later in this paper that the most important factors in the production of toxic symptoms in animals is the blood level of streptomycin and the length of time this blood level is maintained. Tompsett has made similar observations in humans (4). The larger the dose the shorter the period before otic dysfunctions occur, so dosage should always be prescribed in mg. per Kg. of body weight per day.

[1] From the Department of Otolaryngology, College of Physicians and Surgeons, Columbia University, New York, N. Y.

[2] Presented before the Medical Section, as part of the Symposium on *Chemotherapeutics and Antibiotics*, at the 44th Annual Meeting of the National Tuberculosis Association, New York, New York, June 16, 1948.

[3] This study was aided by grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

Another important consideration in streptomycin therapy is the excretory powers of the kidney. Many observers have reported the rapid onset of severe otic dysfunction in patients with high concentrations of drug in the blood as a result of faulty renal excretion. Unexpected otic symptoms could be avoided or minimized by an adequate intermittent check of renal function.

When there is damage to the vestibular system from any cause, the body tries to compensate with the optic and proprioceptive mechanisms. The damage from streptomycin is no exception. More or less compensation is readily achieved by children and most young adults, but is a more serious problem in the older age groups. All groups encounter difficulties in locomotion in the dark or over rough terrain though they may show no disability in bed or on smooth well-lighted hospital floors. So-called "cerebellar exercises" seem to increase the speed of compensation. Putting the patients into classes where several individuals are successfully overcoming their handicap is bound to have an effect on those who cling to their vertigo for various psychological reasons.

The questions which come to all of our minds are where and what is the mechanism of the lesion of streptomycin poisoning and how is it produced. Extensive histological study of the labyrinth, the CNS tracts, and their nuclei have failed to show any definite or consistent lesion which was not also found in control animals. Theoretically the lesion would appear to be located in the vestibular ganglion (Scarpa's) or in the brain stem, but convincing localization of pathology is yet to be demonstrated.

Streptomycin seems to act like an allergen. Some humans and animals do not develop toxic manifestations on the same dosage that affects others. Streptomycin produces hives, rashes, angioneurotic edema or eosinophilia in certain individuals. All of these suggest an allergic type of reaction. Is it possible to combat the toxic effects of streptomycin with antihistamine or, as suggested by Romansky (5), is it possible to prevent the effects by a desensitization regimen? Experiments attempting to answer these question were carried out in the following manner.

Cats had been found to follow very closely the clinical symptoms displayed by humans so they were selected for the study. All animals during the experiment were tested for vestibular function by rotation, using an electric motor-driven turntable. They were rotated ten times in ten seconds. The postrotational nystagmus was observed visually and timed. When 60 r.p.m. no longer elicited a response, they were rotated at 120 r.p.m. for twenty turns and, when this no longer elicited a nystagmus, the vestibular mechanism was considered defunct. Moving pictures of the eyes were made on a good cross-section of animals at various stages of loss. These rotational experiments seemed even more accurate for the determination of quantitative vestibular losses than the Hallpike calorics used in humans. Hawkins has made similar determinations (7). Rotation experiments are more difficult to perform on patients, however.

Gross auditory tests were made using a sharp short noise at 60, 80, 100, and 110 decibels. The noise apparatus was screened from the view of the cats. The auditory response was observed through conditioned palprebral reflexes. When

the response could not be elicited with 110 decibels, the animals were considered deaf.

All animals were given streptomycin[4] by the subcutaneous route in two divided doses a day. Figure 1 is a graph plotting streptomycin dose per kilogram of body weight per day versus days of therapy. (The outside scale is the equivalent dose in grams per day in a 60 Kg. man.) The first heavy line represents the day of lesion in 14 control cats on various streptomycin dosage regimens. Above 400 mg. per Kg. per day the animals died in a very few days in severe dehydration. From this lethal level to one of 66 mg. per Kg. per day (a therapeutic dose) there was a gradual prolongation of the onset of otic dysfunction

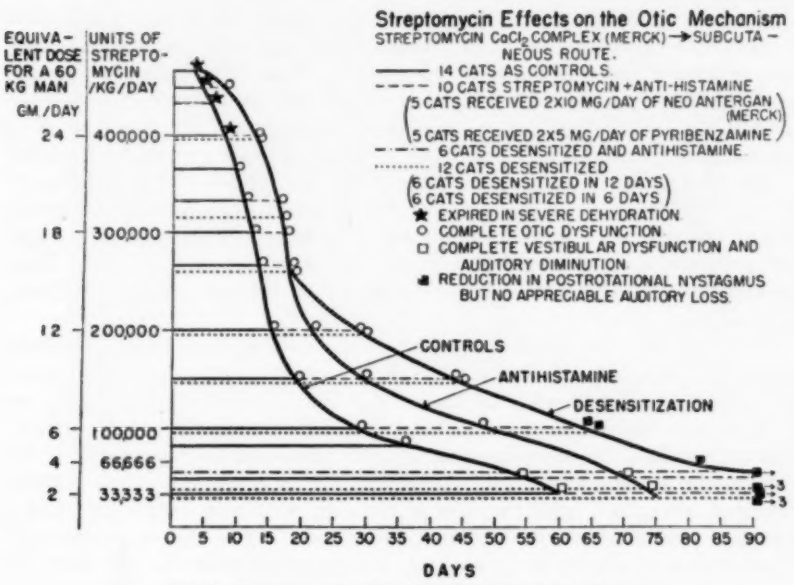
Streptomycin Effects on the Otic Mechanism
STREPTOMYCIN $CaCl_2$ COMPLEX (MERCK) → SUBCUTANEOUS ROUTE.
14 CATS AS CONTROLS.
10 CATS STREPTOMYCIN + ANTI-HISTAMINE
(5 CATS RECEIVED 2X10 MG/DAY OF NEO ANTERGAN (MERCK)
5 CATS RECEIVED 2X5 MG/DAY OF PYRIBENZAMINE)
6 CATS DESENSITIZED AND ANTIHISTAMINE
12 CATS DESENSITIZED
(6 CATS DESENSITIZED IN 12 DAYS
6 CATS DESENSITIZED IN 6 DAYS)
★ EXPIRED IN SEVERE DEHYDRATION
○ COMPLETE OTIC DYSFUNCTION
□ COMPLETE VESTIBULAR DYSFUNCTION AND AUDITORY DIMINUTION
■ REDUCTION IN POSTROTATIONAL NYSTAGMUS BUT NO APPRECIABLE AUDITORY LOSS
EQUIVALENT DOSE FOR A 60 KG MAN GM/DAY
UNITS OF STREPTOMYCIN /KG/DAY
24
18
12
6
4
2
400,000
300,000
200,000
100,000
66,666
33,333
CONTROLS
ANTIHISTAMINE
DESENSITIZATION
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
DAYS

FIG. 1. Streptomycin effects on the otic mechanism.

The points represented by an "O" are the days of complete otic dysfunction. At the lower therapeutic level the points represented by a square are the days of complete vestibular dysfunction but only diminution of auditory function.

The second heavy line represents the day of vestibular loss in 10 animals receiving streptomycin plus antihistamine. Five cats received 20 mg. per day of Neo-antergan. Five cats received 10 mg. per day of pyribenzamine. The approximate 5, 10, and 15 day extension of the day of complete vestibular dysfunction as compared to the control group may be seen. The addition of antihistamine brought on dehydration sooner and in a more severe form than

[4] The streptomycin used in these experiments was the calcium chloride trihydrochloride complex kindly supplied by Merck and Co., Rahway, New Jersey.

streptomycin alone and the animals in general showed greater signs of discomfort, however the time before the otic symptoms developed was prolonged. The last line, which is superimposed on the second line as far as the 250 mg. level, represents the day of complete vestibular dysfunction of 12 cats that were given a desensitization regimen. Briefly, this was done by starting them on a very small dose of streptomycin and bringing them up fairly rapidly to both toxic and therapeutic levels. The desensitization was accomplished in six days at the lower levels and twelve days at the higher more toxic levels. Notice that in high levels there seems to be no protection. An analogous lack of protection is demonstrated by desensitized ragweed sufferers when they are exposed to excessive amounts of the allergen.

All of the desensitized animals ate well, kept themselves clean, and showed no signs of dehydration until the day of practically complete vestibular dysfunction, in spite of the fact that they were receiving doses which made non-desensitized animals in nearby cages very ill. This well-being of the desensitized animals is not understood but may be of prime importance. The combination of desensitization and antihistamine in 6 cats did not produce a superior protection as they also fall in this last line. Of equal interest, besides the prolongation of the onset of symptoms, is the fact that the cats under a desensitization regimen at a therapeutic level (which is equivalent to a human 2 gm. per day dosage) did not after more than 100 days of therapy show any auditory loss. They showed only a slight diminution of response to rotation and no vestibular symptoms.

The toxic effect in cats as well as humans (4) occurs more or less regularly with a certain total dosage except with very low levels. For example, the control series curve approximates a total dosage of 3 Gm. per Kg. of body weight; the antihistamine curve approximates a total dose of 5 Gm. per Kg. of body weight, and the last line, the desensitization regimen, approximates a total dose of 6.5 Gm. in the intermediate level. That the cats on a desensitization regimen tolerated more than twice the dose of the control animals before they had a complete vestibular loss is statistically significant. It is hard to explain just why streptomycin seems to be less toxic in desensitized animals. Whether it is true desensitization or an acquired tolerance is not known, but it is believed that any information about the toxicity of the drug is useful. At the present time we feel justified in at least recommending antihistamine along with streptomycin therapy. The two chief objections to a desensitization regimen are: (*1*) that in acute cases one would not have the time to carry out the desensitization; and (*2*) that in the desensitization period one might give the microorganism involved time to become resistant or one might be allowing resistant strains of bacteria to develop without affecting the nonresistant one soon enough.

If experiments show that bacterial resistance to the drug does not develop in the short desensitization period, then cases of long term therapy for tuberculosis would be greatly aided. The distressing symptoms of vertigo, nausea, and anorexia certainly interfere with the attainment of the rest, good nourishment, and peace of mind required for the successful care of tuberculosis.

Our experience with many tests and several hospitals leads us to recommend the following regimen for the evaluation of otic dysfunction in streptomycin-treated patients.

(*1*) One audiogram in a quiet room before the drug is started. Another audiogram done after one week of therapy and further audiograms should be done after treatment has terminated or if auditory symptoms develop during treatment. Any hospital where large numbers of hearing tests are being done should have an audiometer and a sound-proof booth for testing. The latter can now be obtained, prefabricated, for around $1,000 and a satisfactory one can be built by the hospital carpenter for about $300.00

FIG. 2. The horizontal canals are upright with the patient lying 30° from the horizontal. When the temperature is checked with a thermometer at the meatus, much more accurate tests are obtained.

(*2*) Caloric tests should be done before therapy begins, when and if the symptoms develop, and after treatment is terminated.

a. The best caloric test is the Hallpike test (1) using 30° C. and 44° C. water, controlled by a water bath with a thermostat and stirrer (figure 2).

b. If the Hallpike test cannot be done, a Kobrak test using 5 cc. of ice water with the head back 60° from the vertical is useful. If 5 cc. produces no response, the instillation should be continued up to 50 cc.

(*3*) If there is any indication of vestibular loss by the above tests, the patient should be given exercises in balance, walking along a line, standing on one foot, looking from right to left, turning gently, throwing bean bags about, walking up and down stairs, walking on rough ground, et cetera. A good test of general function is the Glorig mattress test, which consists of walking on a firm mattress first without, then, if possible, with a blindfold (2).

Finally we should like to remind you that it is an ill wind that blows no one any good. Elsewhere one of us (6) has reported the use of toxic effects of streptomycin on the vestibular mechanism in the alleviation of the vertigo in Menier's disease.

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REPEATED TRANSCUTANEOUS AUTOLYTIC TUBERCULIN TESTS IN CHILDREN[1]

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INTRODUCTION

In 1943 (1) it was pointed out and demonstrated by experiment that tubercle bacilli liberate tuberculin (tuberculoprotein) by autolysis. Tuberculin containing a biologically active tuberculoprotein was described originally by Koch as "Old Tuberculin" in the nineties and was obtained by him from a bouillon filtrate after the massive culture of mammalian tubercle bacilli.

The tuberculoprotein was isolated first chemically in crystallizable and precipitable form from the filtrate from a nonprotein simple synthetic medium by Long and Seibert in 1928 (2). The latter preparation, as PPD, was used for accurate intracutaneous test purposes and as a standard tuberculin. Though chemically pure, it did not prove suitable, however, for transcutaneous testing.

The autolytic tuberculin, containing about 10 per cent undenatured biologically active tuberculoprotein and prepared from bacilli free of extraneous materials and chemically unaltered, as well as bacteriologically uncontaminated, possessed properties advantageous to preservation in pure form as well as suited to the preparation of a satisfactory transcutaneous test material (3, 4, 5, 6). It could be suspended in dry form in a suitable inert nonirritating nonwatery liquid containing a cellulose preparation, so the autolytic tuberculin would adhere satisfactorily upon application to the skin. As a result of its hydroscopic nature, it would dissolve by absorbing moisture in close approximation to the skin to permit the immediate action of the tuberculin for the tuberculin allergic hypersensitive test for tuberculosis. The reaction to this test is self-limited by nature and causes no remote focal or systemic reactions, as may occur with intracutaneous or other injection tests for the tuberculin (hypersensitive) reaction, and therefore can be used without reservation even in the tuberculous.

Because of the applicability of this test to repeated use and its simplicity of administration, the question arose whether the test could cause hypersensitiveness to itself when repeatedly applied, since it has been claimed by some that an Arthus phenomenon (hypersensitive allergic condition) can develop to large repeated injections of tuberculin. The writers have been unable to verify this, especially when using bacteriologically uncontaminated and pure tuberculoprotein or autolytic tuberculin in as large amounts as possible for the sensitizing agent. Tuberculin has been used so extensively both experimentally and clinically that errors in results have been numerous and deductions at times have

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lacked critical evaluation. This is well pointed out by an article in 1946 (7) in which it was noted that:

> Tuberculin testing for diagnostic case finding surveys has proved itself reliable by intracutaneous (Mantoux—with proper amount of tuberculin) and transcutaneous techniques (Corper). An interpretation of the significance of reactors and the relation to nonspecificity are presented. The fallacy of evaluating tuberculin tests on the basis of high numbers of reactors is expounded. For case finding surveys, the test which elicits the minimum number of reactors among persons without tuberculous disease is the superior test. A set of criteria based on efficacy as well as reliability is recommended for acceptable tuberculin testing programs.

It is obvious that no materials should be in the test reagent which can produce or elicit a reaction in themselves or can cause evident nonspecific irritation when applied, particularly to the skin of the tuberculous or any normal individual. In order to note whether such reactions can be caused by the adhesive (the cellulose preparation) or the solvent used for applying the autolytic tuberculin for test purposes, the vehicle containing all the ingredients except the autolytic tuberculin powder was applied to the skin of over 100 tuberculous patients (in all states of the disease) at the National Jewish Hospital at Denver and to the skin of over 50 normal individuals. In each case, at least two applications were made in various places on the arms and back. The applications were allowed to remain adherent to the skin until they naturally separated and dropped off, which in over 75 per cent of the cases required from three to six days or more, while the remainder adhered to the skin over two days when properly applied. In no case was there any evidence of either mechanical or chemical irritation, and there was no evidence of maceration of the skin from accumulation of moisture under the application. Even individuals with a sensitive skin tolerated the applications well and without irritation of any kind.

In previous tests on the tuberculous, it was determined that a period of contact of several hours would suffice to produce a positive tuberculin reaction with the autolytic tuberculin "plastotest". As a rule, however, the test reagent should remain in contact with the skin for one to two days after application for best results. The speed of development of the intracutaneous autolytic tuberculin test reaction is about the same as that following intracutaneous tests with specific amounts of pure tuberculin.

OBSERVATIONS

In order to determine whether the transcutaneous autolytic tuberculin "plastotest" (Corper) could cause the development of tuberculin hypersensitiveness, a total of 223 children and adolescents in an orphanage, ranging in ages from 5 to 20 years of age, were tested repeatedly. For the first month, tests were made at weekly intervals and then each month for six months from the initiation of the first test. In all cases, at least two tests were applied either on the forearms, the upper arms, shoulders, or backs, and in many cases four applications of "plastotest" to different sites were made.

In all the 223 children, the period of study covered the six months interval and note was made of any conditions such as other infections, sunburn, et cetera, which might in any way vitiate a reliable test. In many cases a chest roentgenogram was taken for correlation purposes to note particularly whether any evidence existed of parenchymal tuberculous involvement, enlargement of lymph nodes at hilum, Ghon tubercles, or significant calcifications of any kind. In obtaining the roentgenograms, stress was laid particularly on the definitely positive reactors. It is intended to continue testing at the nine month and twelve month intervals after initiation of the tests in all available cases.

The results of the tests are summarized in table 1 in order to note the number of the total 223 children tested who never disclosed a positive reaction to the repeated duplicate (or more) applications of the transcutaneous autolytic tuberculin "plastotest." In table 2, the positive reactors are given more detailed evaluation mainly to note what effect repeated testing would produce on the

TABLE 1

The Results of Repeated Transcutaneous Autolytic Tuberculin "Plastotests" over a Period of Six Months on 223 Orphanage Children

TOTAL CHILDREN TESTED	SEX	TOTAL PERSISTENTLY NEGATIVE	TOTAL DEFINITELY POSITIVE	TOTAL BORDERLINE POSITIVES
223	Males—154 (48 + 81 + 25)*	108	23	23
	Females—69 (30 + 35 + 4)*	54	6	9

* The numerals in parentheses indicate the number of children in each age group: starting with the first numeral, from 6 to 10 years; the second, 11 to 15; and the third, 16 to 20 years.

tuberculin hypersensitiveness of the definitely positive, slightly positive, or questionable cases.

It is noted from the results recorded in table 1 that at least 162 of 223 children repeatedly tested with the transcutaneous autolytic tuberculin "plastotest" consistently gave negative reactions to the test. According to sex, 108 of a total of 154 males and 54 females of a total of 69 remained negative throughout the test period. This represents more than 70 per cent of the total, and 70 per cent of the males, and 78 per cent of the females, who were consistently negative reactors. These figures would appear to justify the conclusion that repeated duplicate applications of the transcutaneous autolytic tuberculin test for prolonged periods does not tend to produce a hypersensitive condition to tuberculin in the recipients of the test.

Whether or not the application of the transcutaneous autolytic tuberculin test is capable of altering consistently the reaction obtained in the positive or slightly positive tuberculin hypersensitive case is disclosed in an examination of the findings obtained in the positive cases and recorded in table 2.

The results recorded in table 2 summarize the serial findings in the positive

TABLE 2

The Variations in the Transcutaneous Reactions to the Autolytic Tuberculin "Plastotest" Applied Repeatedly to Positive or Slightly Positive Tuberculin Reactors over a Period of Six Months

TOTAL NUMBER OF CASES (POSITIVES)	SEX TOTAL (POSITIVES)	DETAILED FINDINGS			
		Definite positive reactors		Slightly positive or questionable reactors	
61	Males 46	23	6 to 10 years	23	6 to 10 years
			12-3-4 to 9* 2 to 5-69-178 67-3589-124 123-4 to 9 1 to 8 78-9-1 to 7 67-49-1 to 358 247-1358-6		1 to 578-6† 468-1 to 357 18-2 to 7 1489-235 to 7 12489-357
			11 to 15 years		11 to 15 years
			23468-157 234-15 to 8-67 8-2 to 7-1 1 to 8 1 to 7-8 1238-4 to 7 4-2356-178 1 to 68-7 2-135 to 9-2 1 to 5-0-678 14 to 68-237-5		14 to 8-23 12458-367 24 to 7-138 568-1 to 47 1 to 4-5 to 8 5 to 7-1 to 48 256-13,478 345-126 to 8 26-13 to 578 2568-1347 2578-1346 4 to 8-123 124 to 8-3
			16 to 20 years		16 to 20 years
			467-358-12 1 to 8 16-78-2 to 5 1 to 8		35 to 8-124 2 to 578-16 1 to 8 3 to 8-12 146 to 8-235
	Females 15	6	6 to 10 years	9	6 to 10 years
			1 to 5-6 to 9 123-4-5 to 8 47 to 9-1236-5		79-1 to 68 5679-1 to 48 5 to 8-1 to 4
			11 to 15 years		11 to 15 years
			1 to 47-568 8-346-1257 8-0-1 to 7		1358-2467 3 to 68-127 13 to 5-26 to 8 3 to 8-12 2356-1478 3467-1258

* The first numeral before the dash indicates the order number of the tests when a positive reaction was obtained, 13 indicating on the first and third test period or 1 to 3 on the first, second, and third tests; while the second numeral following the first dash indicates a slight or questionable positive reaction. When appearing, the third numerals following the second dash were the order of a negative reaction.

† The markings or numerals in this group give only two sets of numerals, the first being the order of the series when a slightly or questionable positive reaction occurred and the second, following the dash, indicating the order of occurrence of a negative test. In all cases either 8 or 9 test periods were performed, the numeral 8 or 9 indicating this.

transcutaneous tuberculin test observations on these children. There is no evidence from these studies that the repeated tests from the transcutaneous autolytic tuberculin "plastotest" material consistently produce in these children either an accentuation of the positive hypersensitive reaction to tuberculin or that it suppresses this reaction. The variations which occur in such serial testing over more than a six month test period are to be attributed rather to the individual variation in the person's reaction powers or to a natural waning or accentuation due to the basic condition in the individual, *i.e.*, the tuberculous infection accountable for the hypersensitiveness to tuberculin. These findings are in entire accord with previous findings with tuberculin tests in which fluctuation in the intensity of the reaction are noted from time to time.

COMMENT

A careful study of the roentgenograms of these children, especially in the positive cases, disclosed in the majority of the definitely positive cases evidences of some type of tuberculous infection, usually lymphatic and less frequently pulmonary parenchymal, although some cases definitely disclosed no evidences of such infection. In the case of the slightly or questionably positive tuberculin-reacting children, the greater number disclosed none or very little evidence of tuberculous infection in the chest. Most of the chest films proved to be essentially negative as recorded by the examining roentgenologist and the clinician examining the film. All films were examined independently by at least three examiners and, as was natural in such a study, there was some disagreement in the readings although evident changes were not missed. Ghon tubercles, hilum and parenchymal shadows were given consideration in reaching a diagnosis. It was also interesting to note in this series that over a period of six months or more a few definitely positive reacting cases gradually were transformed to negative. One case in particular changed abruptly to positive from negative and was found to have experienced a recent definite exposure to tuberculosis. It is also noted that the percentage of positive reactors was approximately equal in the different age periods, and the girls (although fewer were tested) showed a lesser incidence than the boys. Where the reaction was consistently positive throughout the period of test, the evidences as gleaned from the roentgenographic examination were in most cases definitely toward the existence of hilar involvement with or without parenchymal affection. In performing the tests, particular attention should be called to the possible accentuation effects of extraneous conditions, such as the existence of sunburn or multiple abrasions which may lead to the development of false positive nonspecific reactions. These can be excluded readily by careful observation. The effect of acute pyrexias and other acute infectious diseases in altering reaction is too well known to stress further here. In general, the test, as with all tuberculin tests, requires judicious application as well as careful reading of reactions to be of significant diagnostic and survey value. The tuberculin test, a valuable adjunct to diagnosis in medicine, has been too frequently condemned just because of either the use of impure, improper test reagents or careless performance and evaluation of the

test readings. Frequent repeat tests assume importance also in this light, since errors thus are minimized to the advantage of the reliability of any tuberculin test. Duplicate tests, which can be performed easily also with the transcutaneous autolytic tuberculin test to assure better observation as well as proper application, yield more reliable results in the individual case and possess definite advantages.

SUMMARY AND CONCLUSIONS

Frequent and repeated application, in duplicate or more, of the transcutaneous autolytic tuberculin "plastotest" (Corper) extending over more than a six month period does not produce a hypersensitiveness to tuberculin or to the transcutaneous test in children between the ages of 5 to 20 years. Neither does the repeated application of the transcutaneous tuberculin test accentuate or suppress an already existing hyper sensitiveness to tuberculin in children.

SUMARIO

Repetidas Transcutirreacciones Autolíticas a la Tuberculina en los Niños

La frecuente y repetida aplicación, por duplicado o más, de la "plastorreacción" transcutánea autolítica a la tuberculina (Corper) durante un período de más de seis meses no produce hipersensibilidad a la tuberculina o a la transcutirreación en niños de 5 a 20 años de edad. Tampoco acentúa o suprime la repetida aplicación de la transcutirreacción con tuberculina, una hipersensibilidad preexistente a la tuberculina en los niños.

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A SLIDE CULTURE METHOD FOR THE EARLY DETECTION AND OBSERVATION OF GROWTH OF THE TUBERCLE BACILLUS[1, 2, 3]

A Preliminary Report

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INTRODUCTION

The characteristic slow growth of *Mycobacterium tuberculosis* imposes a severe handicap on those engaged in clinical or research work in the field of tuberculosis. This is especially true in the clinical study of tuberculosis because the symptoms, signs, roentgenographic changes, and laboratory findings other than the bacteriologic, are not sufficiently distinctive to be absolutely pathognomonic of the disease. It has been frequently noted that a diagnosis of tuberculosis in the absence of bacteriologic proof is often questionable. In order that the diagnosis be certain, the tubercle bacillus must be demonstrated. The problem is becoming more acute because of the widespread use of the various roentgenographic techniques employed to discover tuberculosis in apparently healthy individuals (1). As a result of these surveys, the proportion of minimal disease coming under observation is increasing (2, 3, 4, 5) and, therefore, the number of patients in whom it is impossible to demonstrate the tubercle bacillus by smear or concentrate is also increasing. Failure to make the proper diagnosis in suspicious cases frequently leads either to the exposure of a nontuberculous individual to the environment of a sanatorium or to a delay in the proper treatment. Such a delay not uncommonly results in progression of the disease (5).

Microscopic examination of a direct smear of material harboring tubercle bacilli is obviously the simplest and most economical method by which the microorganism may be demonstrated. Unfortunately this method is very indelicate, since it has been estimated that it requires 100,000 tubercle bacilli per cc. of smeared fluid in order that the organism may be detected in this manner (6). The fact that there is frequently an insufficient number of organisms in biological fluids for demonstration by direct examination is so well known that it is hardly deserving of comment. It may be useful, however, to cite the figures of Pinner (7) who found that in 533 cases from which the tubercle bacillus was recovered, approximately 60 per cent were "positive" on direct smear or sputum concentrate; 16 per cent more were "positive" on culture of the sputum; 5 per cent were "positive" on direct examination of the gastric concentrate; and 18

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per cent were "positive" only on culture of the gastric contents. These figures are derived from the study of patients in all stages of tuberculosis and adequately demonstrate that direct examinations of sputum smears or concentrates are only about 60 per cent efficient. The inefficiency of the microscopic examination of the sputum is further emphasized by the findings of Decker, Ordway and Medlar (8) who have shown that, in persons with minimal tuberculosis, the tubercle bacillus can be demonstrated by direct examination of the smear in only about 6 per cent. In 269 cases of minimal tuberculosis reported by them, smears were "positive" in 5.9 per cent, whereas culture or guinea pig inoculations demonstrated the presence of tubercle bacilli in 69 per cent of these cases. These figures agree with those reported by other workers (9).

Although it is not within the scope of this paper to enter into the controversy as to which procedure, culture or guinea pig inoculation, is the most useful in detection of the presence of the tubercle bacillus, it should be pointed out that, in spite of the many articles in the literature which advocate the use of culture methods alone (10, 11, 12, 13), from the evidence now available it would seem that when accuracy is the only consideration both methods should be used in each case being studied (14, 15, 16).

The time required for growth to become apparent appears to depend somewhat upon the procedures used and to a greater extent upon the number of organisms in the inoculum. In the ordinary laboratory practice the interval between inoculation and the macroscopic appearance of growth is from 3 to 8 weeks. Exceptionally, colonies first appear as early as 5 days and as late as 3 months following inoculation.

In order to decrease the interval between inoculation and detection of growth, smears have been made of the material scraped from the surface of the culture media at intervals during the period of cultivation. In this manner the time necessary to demonstrate the acid-fast organisms can be considerably shortened. Johnston (17) showed that microscopic examination of the material scraped from the surface of his diagnostic cultures disclosed the presence of the tubercle bacillus in an average time of 20 days, whereas macroscopic growth appeared only after 41.3 days. In this way, 21.3 days were gained. While there is no doubt that the diagnosis can be made more quickly in this manner, it is not practical because of the high rate of contamination (17).

In 1945, a new medium was described by Dubos (18), which was reported by him to produce rapid growth of the tubercle bacillus. Although this medium was designed for purposes other than primary isolation of the tubercle bacillus from pathological materials, it has been used for that purpose by several workers with encouraging results. Foley (19, 20) has reported the successful use of Dubos media for the cultivation of tubercle bacilli from various biological fluids. Goldie has also reported good results with Dubos media in the isolation of tubercle bacilli from sputum (21). Foley noted growth in an average time of 10.6 days, and Goldie reports positive cultures in 8 to 14 days.

Dubos and Middlebrook (22) have recently described a modification of the original Dubos medium, making it more suitable for the primary isolation of

tubercle bacilli. In this paper they state: "Suffice it to say that fairly rapid growth of tubercle bacilli (7 to 15 days) has often been obtained in the liquid media with sputa, spinal fluid, and pleural exudates which have failed to reveal tubercle bacilli on direct microscopic examination."

Sattler and Youmans (23) have been unable to demonstrate any increase in the rate of growth of the tubercle bacillus in Dubos media.

The final evaluation of Dubos medium cannot be made at this time but, even if the most favorable reports are confirmed, there will still remain a relatively long interval between the time the specimen is sent to the laboratory and the time the report is received.

Although it appears that progress has been made in increasing the rate of growth by alteration of the culture medium, the fact remains that the tubercle bacillus grows slowly. For this reason it seemed that it might be profitable to approach this problem by other means, namely the microscopic detection of early growth.

Robert Koch (24), in his paper of 1882 in which he described the tubercle bacillus, also described the preparation of cultures designed for direct microscopic observation of the growth. He accomplished this by coagulating blood serum on watch glasses or in "small hollow glass blocks" in such a way that it remained transparent. In this manner he was able to demonstrate growth of the tubercle bacillus by the end of the first week by means of magnification of 30 to 40 diameters.

Since Koch's report of what may be termed the micro-culture method, we are aware of no further work in the use of this method for the primary isolation of tubercle bacilli until 1941, although others used such a technique as a research tool, utilizing as inocula organisms from pure cultures (25, 26). In 1941, Pryce (27) described a method in principle very similar to that of Koch, although he was apparently unaware of Koch's work. Pryce demonstrated that it was possible to treat a sputum film on glass with acid, thus destroying organisms other than the tubercle bacillus. He advocated preparing the sputum on the floors of Petri dishes or in slide cells made by putting glass rings over the treated smear; he used whole blood as the medium. With this method growth was evident microscopically in from one to six days.

In 1943 Rosenberg (28) attempted to use Pryce's methods but abandoned them. He found the contamination rate excessively high when Petri dishes were used; the slide cell method he found too laborious for large scale use. He devised a method of slide culture in which the smears, prepared on half slides and treated with acid, were placed in water filled bottles with perforated screw caps. The water was removed with a needle attached to a suction pump and replaced with Kirchner's serum-salt mixture. Microscopic growth was visible within a week. This method, although ingenious, is obviously too complicated for general use.

Finally, others have described micro-culture techniques for research purposes. These methods are not adaptable to any large scale application (29, 30).

Thus there was ample evidence that growth of the tubercle bacillus could be

detected microscopically within a week. It appeared, however, that as yet there had been no method of micro-culture devised which did not have serious disadvantages or was not needlessly complicated.

METHOD

From a specimen of sputum[4] six smears are prepared on new glass slides of the usual size (75 by 25 mm.) which are suitable for labeling. The smears differ in no way from those ordinarily used for microscopic examination, except that they are somewhat thicker. The smears are allowed to dry and are then put in a glass rack which in turn is placed in a covered stain dish (commercially obtainable) and submerged in 6 per cent sulphuric acid for 20 minutes (31). After this interval, in order to remove the acid, the rack containing the slides is transferred with aseptic technique successively to three similar dishes filled with sterile water. The slides remain in each of these dishes 10 minutes. They are then placed in the tubes containing the medium (one slide per tube) and incubated at 37°C. On the second, fourth, and sixth days, two cultures are taken out; the slides

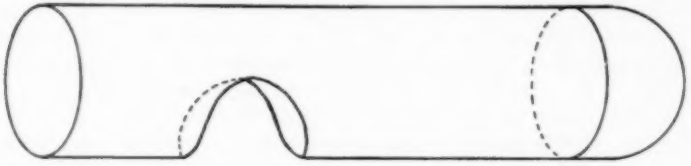

FIG. 1. Culture tube. See text for description. (Drawn by W. A. McGilvray.)

are removed from the tubes, allowed to dry, fixed with heat, labeled, stained, and examined under the microscope. Early in this work half the smears and cultures were stained with auramine. This staining method was abandoned because it was of less value than the Ziehl-Neelsen technique.

Special tubes[5] (figure 1) have been designed specifically for this purpose in such a way that they can be laid horizontally in the incubator. The tubes measure 180 mm. in length, with an inside diameter of 35 mm. One hundred and ten mm. from the closed end of the tube is an indentation 20 mm. in depth, the purpose of which is to prevent the medium from reaching the cotton plug when the tube is horizontal. During incubation a piece of ordinary laboratory glass tubing is placed in the indentation to keep the tubes from rolling. In the horizontal position the maximum opportunity for oxygen diffusion is provided, since the entire sputum film is only slightly beneath the surface of the medium, and, in addition, a large surface of liquid is exposed to air. The amount of medium necessary (20 cc.) is indicated by a mark on the tube.

The medium used for the cultures reported in this paper consisted of 50 per cent Kirchner's solution of electrolytes, glycerol and asparagin (32), and 50 per cent whole citrated blood. In a series now being done, the use of this mixture has been abandoned and Kirchner's basic medium with serum albumin[6] added in a concentration of 0.5 per cent

[4] The specimens of sputum have not been digested or concentrated.
[5] Available through Technical Equipment Corporation, Denver, Colorado.
[6] Bovine plasma fraction V, Armour Laboratories, is satisfactory.

(33) has been used. The growth obtained with the albumin medium appears to be as good as that with the blood, and the fact that the Kirchner albumin mixture is a clear solution is a distinct advantage, since the slides need not be washed upon removal from the tubes before they are dried and fixed. Whether this is the ideal medium for this purpose remains to be determined and studies on this problem are in progress in this laboratory.

Cotton plugs for the tubes are not sterilized in the culture tubes because of the inhibitory effect of distillates from the cotton on the growth of the tubercle bacillus (34). The plugs are placed in glass cylinders of the same diameter (35 mm.) as the tubes and sterilized in the autoclave. At the time the medium is placed in the tubes, the plugs are transferred to them.

RESULTS

All the sputa cultured were from patients whose specimens had at least on one occasion been positive. Two smears were made from each single specimen to be cultured and these were carefully searched for tubercle bacilli.

A total of 140 cultures were made. Twenty-five cultures were made from specimens of sputum which contained numerous bacilli; 39 from specimens containing few bacilli; 40 from specimens which contained rare organisms; and 36 from those in which only 3 or fewer organisms were found in the entire search. The specimens of this last group would probably have been called negative by the standards ordinarily used, since no organisms were seen until the time of searching had been prolonged to one hour or more. This classification of sputa as to whether they contain numerous, few, or rare organisms was made in accordance with that recommended by the Committee on Standard Laboratory Procedure of the American Trudeau Society (35).

Of the total number of cultures upon which this study is based, 37.1 per cent, or 52 cultures, were contaminated. If this high rate of contamination could not be reduced, obviously the method would be of little value. However, many of the factors contributing to the excessive contamination rate have been eliminated by trial and error as work with this new method has progressed. It was necessary, for example, to determine the length of time the smears should remain in acid in order to destroy contaminating organisms. Early in this work, the time was varied from 10 to 45 minutes, but later it was standardized at 20 minutes, since this period proved to be satisfactory. Longer periods of time did not seem to inhibit growth of the tubercle bacillus, but shorter periods appeared to be inadequate. Since all of the cultures are included in the study, the early cultures kept in acid for the shorter lengths of time contributed to the high rate of contamination reported for the series.

The use of the glass rack mentioned above for transferring the slides from one container to another was begun during the course of the work because it was felt that the possibility of air contamination would be less than when the slides were transferred individually. Again, the results of the earlier method are included.

Perhaps the most important factor in the high contamination rate is the fact that during most of the work reported in this paper it was necessary to share a laboratory where a great deal of other work was being done, including animal

autopsies. When a clean workroom became available, the contamination rate dropped markedly.

Following these various improvements, the rate of contamination in about 100 cultures of a series to be reported later is approximately 1.5 per cent.

Of uncontaminated cultures (88), growth was present in 89.8 per cent. This figure is without regard to the length of time the culture was incubated; that is, 2, 4, and 6 day cultures are included.

TABLE 1

Summary of Results (Uncontaminated Cultures) with Slide Culture Method for Detection of M. tuberculosis

Over-all percentage of cultures showing growth is shown and in addition the results in relation to the time of incubation (uncontaminated cultures)

	NUMBER	GROWTH PRESENT		GROWTH ABSENT	
		Number	Per cent	Number	Per cent
Total	88	79	89.8	9	10.2
2 days	29	26	89.7	3	10.3
4 days	34	28	82.4	6	17.6
6 days	25	25	100	0	0

TABLE 2

Slide Culture Method for Detection of M. tuberculosis

The number of organisms seen on direct smear of the specimen is correlated with growth after two, four and six days' incubation (uncontaminated cultures)

TIME INCUBATED	ORGANISMS SEEN IN DIRECT SMEAR							
	Numerous		Few		Rare		3 or less	
	Growth	No growth	Growth	No growth	Growth	No growth	Growth	No growth
days								
2	3	0	4	2*	9	1	10	0
4	5	0	7	0	6	3*	10	3†
6	4	0	7	0	8	0	6	0

* These were the cultures stained with auramine (see text).

† In two of these faulty staining technique may account for the failure.

In table 1 may be seen the results with uncontaminated cultures separated into 2, 4, and 6 day groups. Growth was present in 89.7 per cent of 2 day cultures; in 82.4 per cent of 4 day cultures; and in 100 per cent of those incubated for 6 days. The fact that growth was detectable in such a high percentage of 2 day cultures indicates that routine examination at that time is far from unproductive.

Table 2 shows results in relation to the number of organisms seen on examination of the direct smears of the sputum specimens.

Of the 9 (10.2 per cent) cultures which failed to show growth in spite of being uncontaminated, 2 were stained with a faulty technique; 5 were stained with auramine, and in each instance cultures from the same specimen incubated the

same length of time and stained with carbolfuchsin showed growth; the remaining two were 2 and 4 day cultures. In 20 of the contaminated cultures, growth of tubercle bacilli was present.

The appearance of the colonies of *M. tuberculosis* growing in the slide cultures may be seen in figures 2 through 7.

DISCUSSION

Although it has been known since 1882 that growth of the tubercle bacillus could be detected after one week's incubation by means of the low power lenses of the microscope and attempts have been made to utilize this fact, apparently none of the methods thus far devised is sufficiently practicable for routine use.

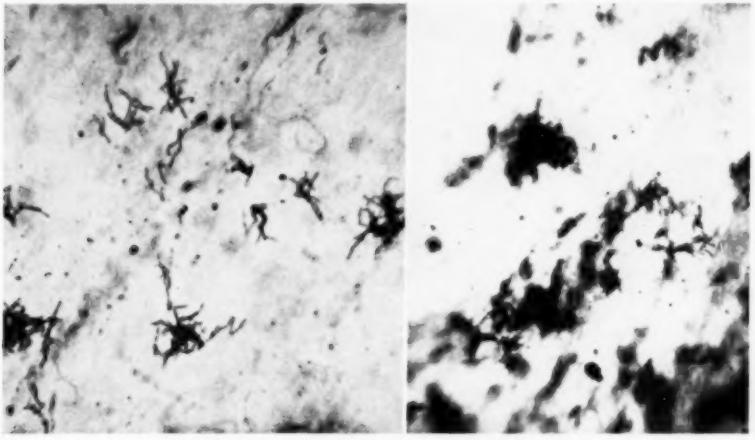

FIG. 2. (Left) Two day culture (6 x 90), Ziehl-Neelsen stain
Small colonies are visible on the background of the sputum film.

FIG. 3. (Right) Three day culture (6 x 90), Ziehl-Neelsen stain
This illustrates the marked beading of the organisms which is occasionally seen.

The present method is reported because it is believed to be sufficiently simple for general use by laboratory technicians. All the equipment required is inexpensive and commonly used in laboratories, with the exception of the tubes which can now be obtained through a commercial supply house. The technique is not complicated and it is believed that it may be feasible to simplify it even further. For instance, it is possible that the time the slides remain in water can be shortened. We have perhaps been overcautious in our effort to be certain that the acid was completely removed.

Because of the characteristic morphology of the colonies, they are easily identified. After six days' incubation they are readily visible with the low power of the microscope (6 x 10). The low power is also usually adequate for the examination of the 4 day culture. Occasionally, however, it is necessary to use greater magnification (6 x 45). It is necessary to use the high dry lens to examine

the 2 day culture, and not uncommonly the oil immersion lens is required (6 x 90). On some occasions cultures have been examined after 24 hours' incubation and

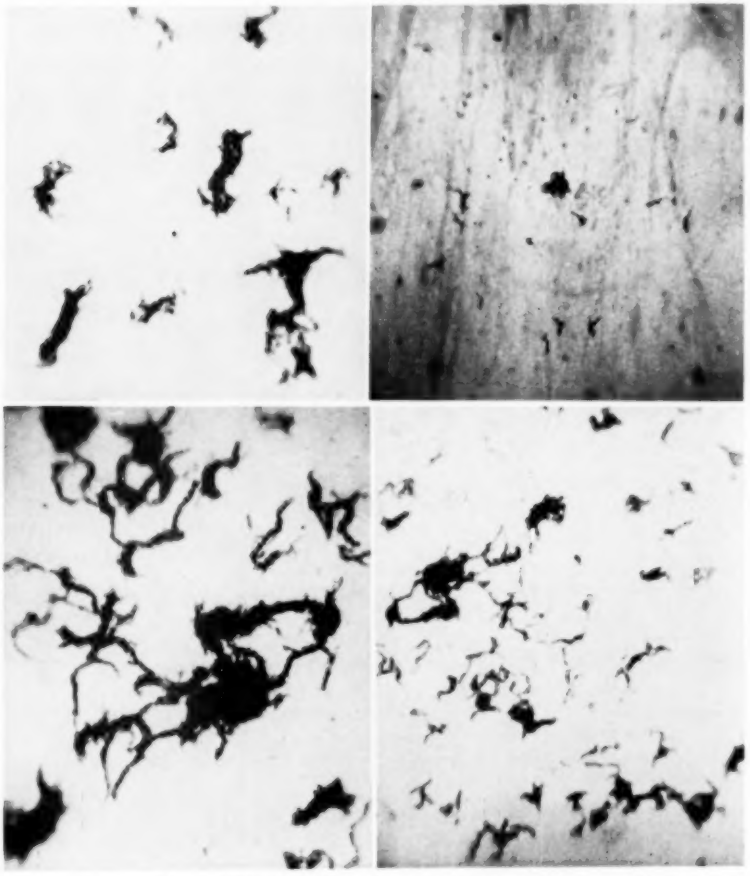

FIG. 4. (Upper left) Four day culture (6 x 90), Ziehl-Neelsen stain
No sputum is visible in this field.

FIG. 5. (Upper right) Four day culture (6 x 45), Ziehl-Neelsen stain
This illustrates how easily colonies of this age are seen with low magnification.

FIG. 6. (Lower left) Six day culture (6 x 90), Ziehl-Neelsen stain without counterstain.

FIG. 7. (Lower right) Six day culture (6 x 45), Ziehl-Neelsen stain without counterstain.

growth has been apparent. One quickly becomes proficient in the study of slide cultures, but it is advisable at the start of work with this method to culture a strongly "positive" sputum in order to become familiar with the appearance of the colonies (figures 2 through 7).

We are testing the diagnostic efficiency of the slide culture method in another laboratory by comparing its accuracy with that of a conventional culture method. Work is also being done in this institution in an effort to adapt the technique to the determination of streptomycin sensitivity of the tubercle bacillus directly from the sputum, without preliminary isolation.

With this technique, further information about the morphology and growth patterns of the acid-fast bacilli may be obtained and comparisons between various pathogens and between pathogenic and nonpathogenic organisms made. The development of colonies can be closely followed by serial termination of growth at short intervals. This method will also provide more precise information as to the effects on growth produced by alterations of media.

SUMMARY

A technically simple method by which *M. tuberculosis* can be cultivated directly from sputum on slides is described. Growth of the microorganism is evident in from one to six days.

Although the experience is too limited to justify final conclusions, the results suggest that this may be a useful method for the rapid diagnostic demonstration of the tubercle bacillus. It is believed that, because of its simplicity, this method will be a useful research tool.

SUMARIO

Técnica del Cultivo en Placas para el Descubrimiento Temprano y Observación del Crecimiento del Bacilo Tuberculoso: Comunicación Preliminar

El método, técnicamente sencillo, aquí descrito, permite cultivar el *M. tuberculosis* directamente del esputo, en placas. Las colonias son visibles en uno a diez días.

Si bien las observaciones son demasiado limitadas para justificar conclusiones definitivas, los resultados indican que esta técnica puede ser útil para el rápido hallazgo del bacilo tuberculoso, con fines de diagnóstico. Parece que, debido a su sencillez, también va a resultar útil para investigación.

Acknowledgments

The authors wish to acknowledge the assistance of Dr. Richard Thompson, Professor of Bacteriology, who made the suggestion which led to this work and loaned them space and the facilities of his department.

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THE PHARMACOLOGIC AND CHEMOTHERAPEUTIC ACTION OF SOME NEW SULFONES AND STREPTOMYCIN IN EXPERIMENTAL TUBERCULOSIS[1, 2]

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INTRODUCTION

In 1940 Rist and associates showed that 4,4′-diaminodiphenylsulfone (DDS) had a favorable effect in experimental tuberculosis in rabbits infected with an avian strain (1). Later the same was shown to be true for experimental tuberculosis in guinea pigs infected with a human strain (2). Since then many attempts have been made to develop compounds chemically related to or derivatives of DDS with the objectives of decreasing toxicity and increasing activity. The first objective has been realized, for there are now many derivatives of DDS that are less toxic than the parent compound. The second objective has not met with as much success.

Shortly after the discovery of streptomycin, it was shown that the use of this antibiotic, together with suitable sulfones, resulted in mutual potentiation of action so that the combined effect in experimental tuberculosis was greater than the sum of effects from the two drugs when used alone (3). Hence the further need of sulfones of low toxicity, if only of moderate activity, as supplementary aids in the treatment of tuberculosis with streptomycin.

Generally speaking there are two types of derivatives of DDS: (*1*) The disubstituted derivatives, in which a hydrogen in each of the two amino groups is replaced by a substituent, *e.g.*, Promin, Diasone and the more recent Sulphetrone (4); and (*2*) the monosubstituted derivatives in which one of the amino groups remains free. In the latter class, the alkyl monosubstituted derivatives have been found to be the least toxic and most effective (5). These compounds, however, are characterized by low absorbability. Further experimentation showed that the introduction of a hydroxyl group into the alkyl substituent increased absorbability without sacrifice with respect to toxicity and activity.

The present paper is a report on the actions of the newer disubstituted derivative Sulphetrone (4)[4] and the monosubstituted 4-amino-4′-β-hydroxyethylaminodiphenylsulfone (6)[5] synthesized in this laboratory and hereafter designated

[1] From the Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Maryland.

[2] Presented at the Tuberculosis Study Section Meeting, September 21, 1948.

[3] Watumull Foundation Fellow.

[4] 4,4′-Bis (γ-phenyl-n-propylamino) diphenylsulfone-tetrasodium sulfonate. Supplied by Dr. Edwin J. de Beer, the Wellcome Research Laboratories.

[5] 4-Amino-4′ -β -hydroxyethylaminodiphenyl sulfone crystallizes as dimorphic forms melting at 130.5–131.5°C. and 143.5–144.5°C. The two forms have shown about the same absorption, toxicity and chemotherapeutic activity, the latter tested in experimental pneumococcus infection in mice. We are indebted to Dr. Augustus Gibson and Dr. Max Tishler of Merck & Company for the supply of a large quantity of the compound.

hydroxyethyl. For comparison, data are also included on the older and better known disubstituted derivative Promin and the parent substance DDS. Promin and Sulphetrone are water soluble and can be administered parenterally; DDS and hydroxyethyl are only slightly soluble in water and are usually administered orally.

EXPERIMENTAL

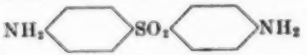
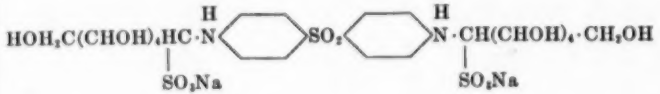
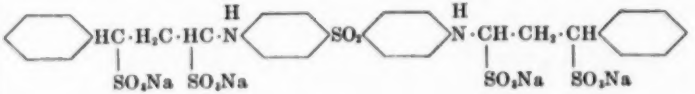
Table 1 shows the structural formulas of these compounds and their DDS equivalent. Hydroxyethyl on direct diazotization at room temperature, according to the Bratton and Marshall technique, represents 46 per cent of DDS, close to the theoretical value. Promin and Sulphetrone are 15 and 13 per cent, respectively, showing their instability, for theoretically, having no free amino group, they should have no diazotization value. Both of these compounds in 0.25 *N* hydrochloric acid containing some trichloroacetic acid are decomposed at 100°C., the diazotization values being short of the theoretical values, probably because of the presence of impurities. Under similar conditions, there is no splitting of the molecule of hydroxyethyl. This was demonstrated by keeping a solution of 0.3 Gm. of hydroxyethyl in 0.25 *N* hydrochloric acid containing 2.5 per cent trichloroacetic acid at 100°C. under a reflux condenser for one hour. Concentration and neutralization of the solution by sodium hydroxide yielded 0.3 Gm. of pure hydroxyethyl, identified by its melting point and a mixed melting point determination with the authentic compound.

Figure 1 illustrates the values obtained on direct diazotization of DDS and Promin at room temperature and after acid hydrolysis, as described in a previous report (7). Plotting light absorption obtained with a Fisher electrophotometer against concentration in mg. per 15 cc., curve 1 represents values obtained for DDS. The direct values and those obtained after heating were identical. Curve 2 represents the values obtained for Promin on direct diazotization and curve 3 after acid hydrolysis. When the blood or urine of an animal receiving DDS is analyzed by the two procedures, the values are identical, as expected if the DDS is unaltered in the animal body. When the blood or urine of an animal receiving Promin by oral administration is analyzed in the same manner, the values are also identical which suggests that Promin is metabolized in the body to DDS. Sulphetrone gives similar results. The properties of a metabolite isolated from the urine of man receiving the hydroxyethyl indicate that this is not degraded to DDS. This metabolite was obtained, by a method to be described later, in solid condition melting at 170 to 174°C. and showing diazotization and coupling with the Bratton-Marshall reagents. A mixture of this material with authentic DDS crystals of m.p. 176 to 177°C. melted at 142 to 145°C., which shows that the metabolite is not DDS. Although the quantity of material available was insufficient for complete purification and identification, the possibility of the oxidation of hydroxyethyl in the human body to the previously described glycine derivative (6) is mentioned[6].

[6] Using a similar technique of isolation DDS was recovered nearly quantitatively and identified by melting point when added to normal urine. In like manner DDS was also

TABLE 1

Structural Formulas and Analyses of 4,4'-diaminodiphenylsulfone (DDS) and Three Derivatives

		SHS EQUIVALENT		
		Theory	Analysis	
			Direct	After 1 hour at 100°C.
DDS		100	100	100
Promin		31	15	24
Sulphetrone		27.8	13	22
Hydroxyethyl		42.4	46	—

Toxicity studies: Data in table 2 give the approximate LD_{50} in rats on oral administration as 1.0 Gm. per Kg. for DDS, 3 to 4 Gm. per Kg. for Promin and more than 4 Gm. per Kg. for Sulphetrone and hydroxyethyl. On intravenous injection the LD_{50} for Promin is between 3 and 3.5 Gm. per Kg., for Sulphetrone 4.5 Gm. per Kg. In terms of DDS equivalent, the toxicity of the two disub-

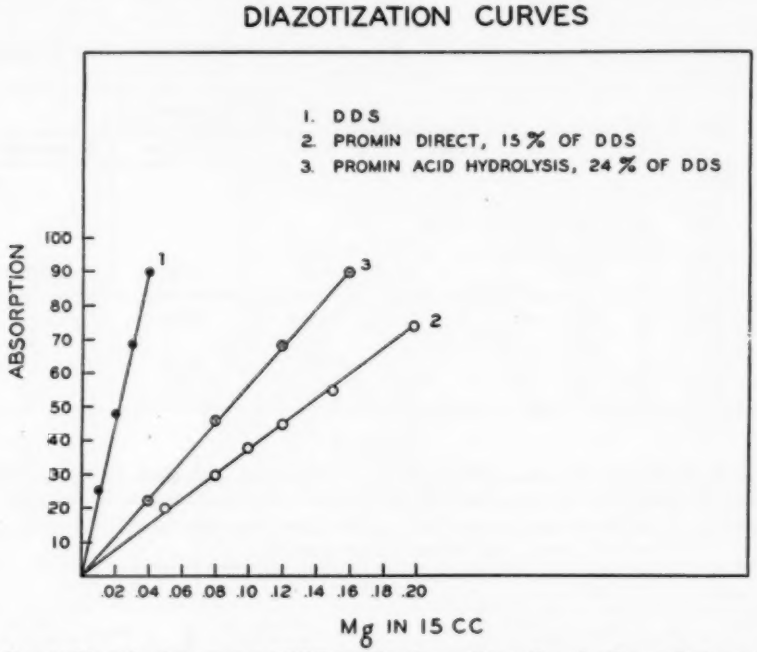


FIG. 1. Diazotization curves of DDS and Promin on direct diazotization and after acid hydrolysis at 100°C. for one hour. Values for DDS are identical; two sets of values are obtainable with Promin. Similar analysis of blood and urine of animals receiving Promin orally yield nearly identical values as with DDS suggesting the degradation of Promin to DDS.

stituted derivatives is almost identical and considerably greater than that of hydroxyethyl.

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recovered from urine of rabbits receiving DDS and identified by melting point. Moreover a substance with m.p. 199 to 200°C. was isolated from the urine of a cat receiving hydroxyethyl and when mixed with the pure glycine derivative

it gave a m.p. of 201 to 202°C. The pure glycine derivative melts at 202 to 203°C. This isolated material also gave a diazotization curve identical with that of the glycine derivative. These experiments will be described in detail in a later publication.

The chronic toxicity in rats when fed in a semisynthetic diet is shown in table 3. The tolerated concentration of DDS is 0.2 per cent of the diet. At this concentration the blood levels were 1.2 to 2.6 mg. per 100 cc. Promin is tolerated at a concentration of 0.8 per cent, almost exactly the same as DDS when computed on the basis of DDS content. Sulphetrone under the same conditions

TABLE 2

Acute Toxicity of 4,4'-diaminodiphenylsulfone (DDS) and Derivatives in Rats Weighing 125 to 175 Gm.

DOSE	ROUTE	MORTALITY, NUMBER DIED/NUMBER USED			
		DDS	Promin	Sulphetrone	Hydroxyethyl
Gm/Kg					
0.8	Oral	3/10			
1.0	Oral	*6/10*			
3.0	Oral		4/10		
4.0	Oral		*6/9*	2/10	0/10
3.0	I.V.		3/10	0/10	
3.5	I.V.		*7/10*		
4.0	I.V.		5/5	2/10	
4.5	I.V.			*7/15*	

TABLE 3

Chronic Toxicity of 4,4'-diaminodiphenylsulfone (DDS) and Derivatives in Young White Rats Weighing 60 to 80 Gm. when Fed in the Diet in the Concentrations as Indicated

Mortality in relation to number used (M) and blood levels mg. per 100 cc. (BL) at the end of the experimental period

PER CENT DRUG IN DIET	DDS			PROMIN			SULPHETRONE		
	M	Days	BL Mg. per 100 cc.	M	Days	BL Mg. per 100 cc.	M	Days	BL Mg. per 100 cc.
0.2	*0/10*	50	1.2-2.6						
0.3	10/10	2 to 11							
0.5	10/10	4 to 10							
0.8				*0/10*	46	9 -26			
1.0	10/10	1 to 6		4/10	11 to 38				
1.5				8/10	3 to 38	6.4-22.0			
3.0				10/10	3 to 43		*0/9*	105	10.8-34.0
5.0							2/10	46*	12 -33

* Subnormal growth.

is much less toxic for it is tolerated in concentrations of 3 to 5 per cent. The blood levels at the high concentration of Sulphetrone in the diet were not significantly higher, however, than those of Promin at the concentration of only 0.8 per cent in the diet, suggesting that the relatively low toxicity of Sulphetrone may be due to lower absorbability.

The same appears to be true from the data on cumulative toxicity in guinea

pigs, shown in figure 2. Three groups of 10 guinea pigs each were fed by stomach tube 1.0 Gm. per Kg. twice daily of Promin, Sulphetrone and hydroxyethyl, respectively. All the animals in the Promin group were dead after 10 doses. Only one of the 10 in the Sulphetrone group died after 14 doses and 2 in the hydroxyethyl group after 20 doses. All the others survived 30 doses. However, blood level determinations made at comparable periods after the administration

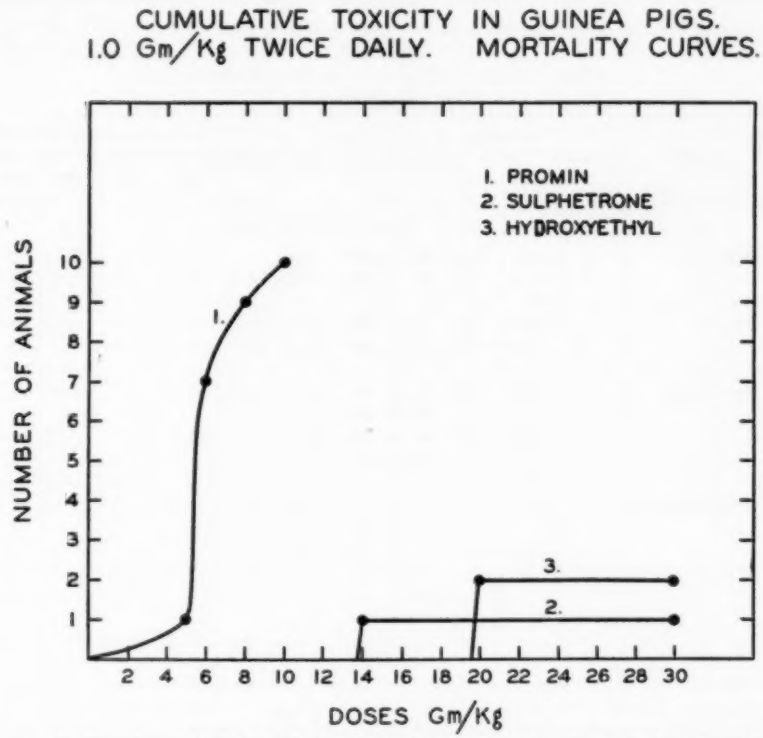

FIG. 2. Cumulative toxicity of Promin, Sulphetrone and 4-amino-4'-β- hydroxyethylaminodiphenylsulfone in guinea pigs. One Gm. per Kg. twice daily orally.

of the respective compounds indicated much higher values for Promin than Sulphetrone or hydroxyethyl. The average blood levels in the Promin group were 26 to 48 mg. per 100 cc., Sulphetrone 5 to 10 mg. per 100 cc. and the hydroxyethyl group 1 to 7 mg. per 100 cc.

Hemoglobin determinations showed a reduction on an average of from 14.3 to 12.0 Gm. per 100 cc. during treatment for the Promin group, from 14.8 to 12.5 Gm. per 100 cc. for the Sulphetrone group and from 14.9 to 13.3 Gm. per 100 cc. for the hydroxyethyl group.

In table 4, data of the chronic toxicity of some of these compounds in cats are

given. The compounds were administered daily, incorporated in a ration of 100 to 200 Gm. ground lean beef, for voluntary consumption. Two animals in the first group received 50 mg per Kg. DDS, the second group of 2 animals received the DDS equivalent as Promin, the third group of 2 animals received first the DDS equivalent as Sulphetrone followed by twice the DDS equivalent and the last 3 animals in the fourth group were given from 1 to 4 times the DDS equivalent as the hydroxyethyl compound. The DDS animals lost weight

TABLE 4

Chronic Toxicity and Effect on Blood Pigments in Cats
Oral Administration

COMPOUND	NUMBER	WEIGHT KG.		DOSES	TOTAL DOSE	BLOOD LEVELS	BLOOD PIGMENTS, GM. PER 100 CC.			
		Initial	Final				Total Hb	HbO_2	MHb	SHb
				mg/Kg	*Gm/Kg*	*mg per 100 cc.*				
DDS	1	4.2	2.3	30 × 50*	1.5					
	2	3.5	1.7	65 × 50	3.25	3.0	11.7	8.6	0.9	2.8
Promin	3	3.2	2.7	70 × 200	14.0	16.0	12.1	10.0	0.9	1.5
	4	3.4	1.5	70 × 200*	14.0	19.5	11.7	8.9	1.1	2.1
Sulphetrone	5	2.9	3.2	68 × 200 16 × 400	20.0	14.0	12.6	9.7	1.0	2.5
	6	2.9	3.5	68 × 200 16 × 400	20.0	11.6	15.5	13.2	0.9	1.9
Hydroxyethyl	7	3.2	2.7	34 × 100 14 × 400	9.0	3.0	12.6	11.9	0.8	1.2
	8	3.9	3.7	5 × 100 29 × 200 16 × 400	12.7	3.5	13.0	11.4	0.7	1.1
	9	3.7	3.2	5 × 100 29 × 200 13 × 400	11.5	2.4	13.3	13.2	0.5	0.5
Controls, average of 9 determinations in 5 normal cats.....							14.3	13.9	0.3	0.4

* Died.

rapidly, developed anorexia, frequently refusing to consume their ration, and thus failed to ingest the drug. One of the animals died after 30 doses. The Promin animals tolerated treatment somewhat better but on the whole displayed similar effects and one died after 70 doses. The Sulphetrone and hydroxyethyl animals accepted the drugs well, showed no visible effects and appeared in good health. Spectrophotometric analysis of blood pigments towards the end of the observation period by the method of Evelyn and Malloy (8) revealed more methemoglobin and sulfhemoglobin in the DDS, Promin, and Sulphetrone animals than in the hydroxyethyl animals. The reduction in oxyhemoglobin

in relation to the total hemoglobin was also more pronounced in the first three groups.

Blood level determinations made uniformly at about 18 hours after feeding, at the time when the animals were receiving 200 mg. per Kg. Sulphetrone and 100 to 200 mg. hydroxyethyl, indicated much higher drug concentrations in the Promin and Sulphetrone groups than in the hydroxyethyl group.

Summing up the data on toxicity it may be concluded that Promin is about as toxic as DDS when dosage is computed on the basis of DDS equivalent, while Sulphetrone and hydroxyethyl are much less toxic. Since the blood levels were

TABLE 5

Blood Levels in mg. per 100 cc. in Guinea Pigs at Various Intervals Following the Oral Administration of Three Sulfone Derivatives in Doses of 0.1 to 2.0 Gm. per Kg.

HOURS	DOSE	PROMIN	SULPHETRONE	HYDROXYETHYL
	Gm/Kg	*Mg. per 100 cc.*	*Mg. per 100 cc.*	*Mg. per 100 cc.*
1	0.1	2.0	2.1	1.2
3		2.4	2.6	0.8
5		2.7	3.0	0.5
24		1.1	trace	0
1	0.5	3.6	2.4	2.4
3		8.0	4.0	3.2
5		13.0	4.6	2.9
24		2.8	2.6	0.7
1	1.0	7.3	4.3	3.4
3		9.1	5.5	4.0
5		9.0	6.3	5.0
24		13.0	3.6	1.6
1	2.0			6.1
3				7.5
5				4.7
24				2.4

generally lower for the last two compounds the question of absorption, tissue distribution, and excretion was considered next.

Metabolism studies: Data on the absorption of these compounds from the gastrointestinal tract of the guinea pig, as judged by drug concentration in the blood, are given in table 5. Blood level determinations were made at various intervals up to 24 hours following a single dose of 0.1 to 1.0 Gm. per Kg. of Promin and Sulphetrone and up to 2.0 Gm. per Kg. hydroxyethyl. The highest blood levels were obtained with Promin, the lowest with hydroxyethyl. In rabbits, given 0.5 Gm. per Kg. of either Promin or Sulphetrone orally, the Promin blood levels were more than twice as high as the Sulphetrone, as shown in figure 3.

To determine the relative retention of Promin and Sulphetrone, each was

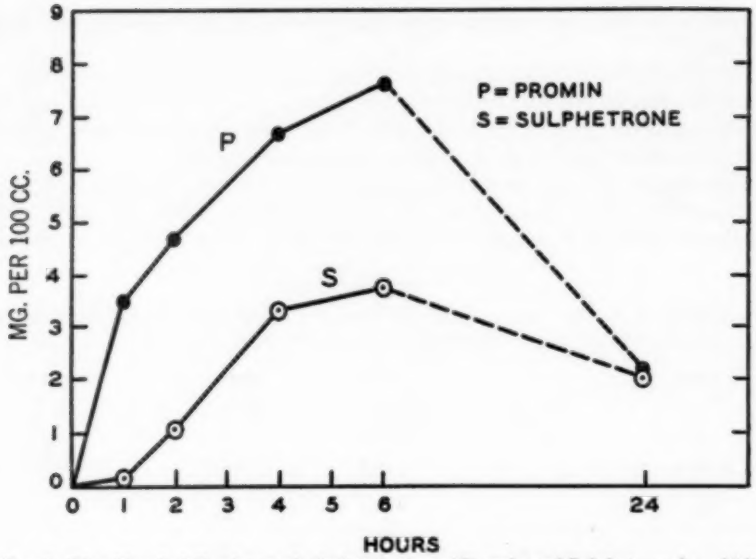

FIG. 3. Blood levels following oral administration of Promin and Sulphetrone in rabbits. Significantly higher blood levels of Promin suggest better absorption.

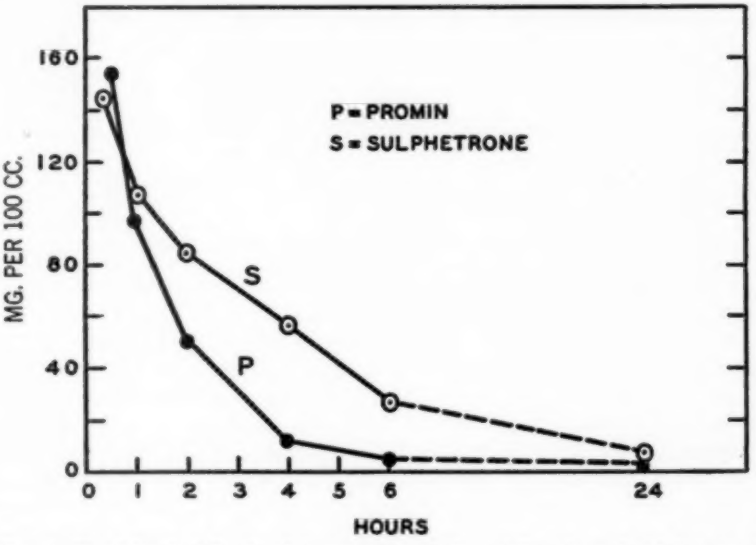

FIG. 4. Blood levels following intravenous injection of Promin and Sulphetrone in rabbits. Results indicate better retention of Sulphetrone.

injected intravenously in rabbits in doses of 0.5 Gm. per Kg. and blood levels determined at stated intervals during 24 hours, with the results as shown in figure 4. It is evident that both drugs leave the blood stream rapidly, though Sulphetrone appears to be retained somewhat better than Promin.

Further data on the metabolic fate of Promin and Sulphetrone are given in figure 5. Both drugs are excreted in the urine to the extent of about 60 per cent of the dose administered when given intravenously. Moreover, the acid hydrol-

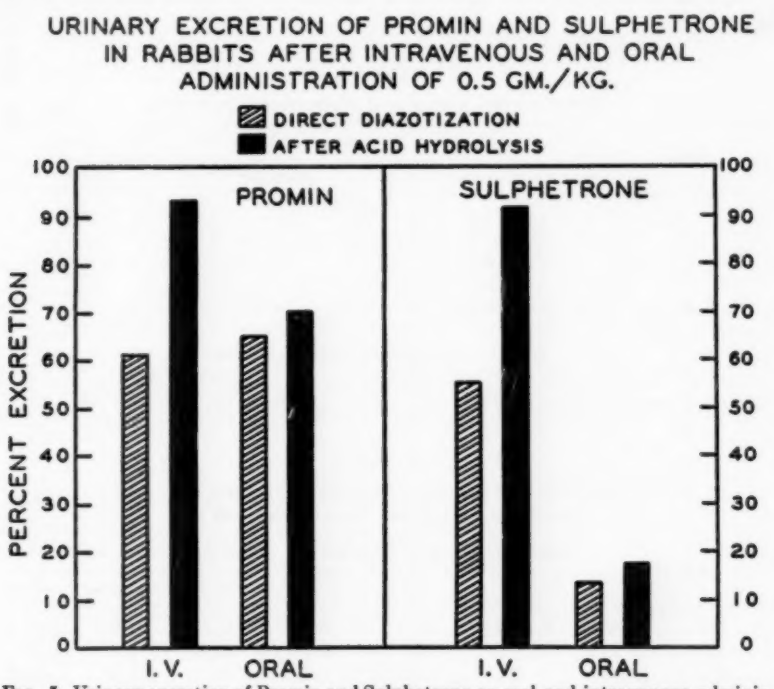

FIG. 5. Urinary excretion of Promin and Sulphetrone on oral and intravenous administration in rabbits in relation to dose administered and metabolic fate. Results indicate degradation of both to DDS on oral administration and poorer absorption of Sulphetrone.

ysis diazotization values are higher for both drugs compared with direct diazotization values. This suggests that both compounds are excreted to a large extent, unchanged, when given intravenously. On oral administration the urinary excretion of Promin is still about 60 to 70 per cent of the dose administered while in the case of Sulphetrone only 15 per cent of the dose given is recoverable in the urine, indicating much poorer absorption. The acid hydrolysis diazotization values on oral administration are nearly identical with the direct diazotization values in both drugs suggesting their degradation to the parent substance.

The urinary elimination of hydroxyethyl in relation to the dose administered was studied in rabbits and in man. The experiments in rabbits are summarized in table 6 and show that, regardless of the dose administered orally, nearly 50 per cent is recoverable from the urine in 24 hours. An experiment in 3 human subjects to whom the compound was administered orally in 2 Gm. doses three times during the day (approximately 0.1 Gm. per Kg.) gave results, shown in table 7, which almost paralleled the results obtained in rabbits. From 40 to 60 per cent of the dose administered was excreted in the urine, and most of it during the first day.[7]

TABLE 6

Absorption and Excretion of Hydroxyethyl in Rabbits

HOURS	BLOOD LEVELS MG. PER 100 CC.		
	0.1 Gm/Kg	0.5 Gm/Kg	1.0 Gm/Kg
1	1.2	2.3	3.7
2	0.9	1.9	3.7
4	0.9	1.9	4.2
6	0.7	1.5	3.7
24	trace	trace	1.3
Urinary excretion, percentage of dose			
24	45	46	52

TABLE 7

Blood Levels and Urinary Excretion of Hydroxyethyl in Man

Dose: 2 Gm.—8:00 a.m., 12:00 m. and 4:00 p.m.

SUBJECT	BLOOD LEVELS, MG. PER 100 CC., HOURS				URINARY EXCRETION, PER CENT OF DOSE		
	2	4	7	24	First day	Second day	Total
HB	0.4	0.5	0.9	0.6	43	0.9	43.9
AK	0.5	0.6	1.0	0.7	55.8	4.6	60.4
GK	0.7	0.6	0.9	0.6	38.5	1.5	40.0

The low blood levels compared with the relatively high urinary excretion following the oral administration of hydroxyethyl suggested the possibility of a preferential distribution of the compound in the tissues. This was studied in several guinea pigs after a single dose of 0.5 Gm. per Kg., and the average results are given in figure 6. The lowest concentrations, estimated as hydroxyethyl, were found in the erythrocytes; the plasma showed somewhat higher concentrations than whole blood; and the liver, kidney, spleen, and lungs gave values ranging from 3 to 10 times those of whole blood. Estimations of the compound

[7] We are indebted to Doctors Howard M. Payne of Freedman's Hospital and Philip Kilbourn of the Tuberculosis Division, Public Health Service, for their cooperation.

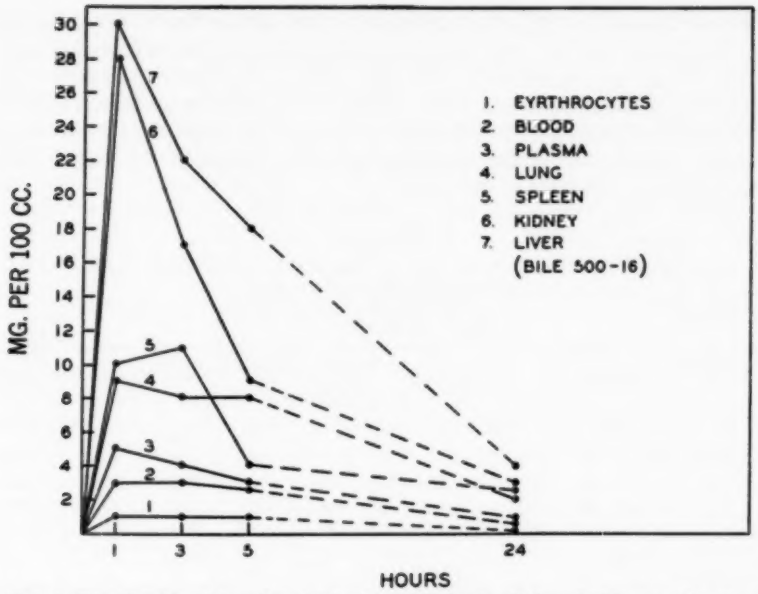

FIG. 6. Distribution of 4-amino-4′-β-hydroxyethylaminodiphenylsulfone in the tissues of guinea pigs. Average of 3 to 4 animals. Highest concentration in liver and kidney, lowest in erythrocytes.

TABLE 8

Chemotherapeutic Activity in Experimental Pneumococcus Type I Infection in Mice

TOTAL DOSE	ROUTE	PER CENT SURVIVAL OF INFECTED MICE	TOXICITY LD_{50} IN NORMAL MICE	LD_{50}/SD_{50}
		Hydroxyethyl		
Gm/Kg			*Gm/Kg*	
4.0	os	73	>8.0	>8/3
3.0	os	*51*		
4.0	sc	75		
3.0	sc	68	>4.0	>4/2
2.0	sc	*50*		
1.0	sc	30		
		DDS		
1.0	os	92		
0.6	os	69	0.25	
0.4	os	*41*		
1.0	sc	87	0.25	1/2
0.5	sc	*49*		

in the bile indicated as high a concentration as 500 mg. per 100 cc. at 1 to 3 hours after the administration of the compound and 16 mg. per 100 cc. 24 hours later.

Thus it may be concluded that Promin is better absorbed than Sulphetrone; that both drugs are apparently metabolized to DDS when given orally; that the low blood levels following the oral administration of hydroxyethyl are not due to poor absorbability but rather to a preferential localization of the compound in certain tissues and organs of the body.

TABLE 9

Activity in Guinea Pig Tuberculosis. 0.5 Mg. H37 Rv I.P. Treatment Day after Infection and Continued 80 Days

DATE OF INOCULATION	NUMBER OF ANIMALS	THERAPY, MG/KG/DAY		MORTALITY PER CENT	AVERAGE WEIGHT GAIN	AVERAGE T.B. INDEX*	CHEMOTHERAPEUTIC EFFECTIVENESS†
		Sulfone orally	Streptomycin intramuscularly				
					Gm		
November, 1947	10	0	0	80	40	15.5	
	10	Hydroxyethyl-300	0	10	118	6.2	2.5
	10	0	20	10	276	1.5	10.3
	10	Hydroxyethyl-100	20	0	304	0.8	19.4
June, 1948	15	0	0	86	13	12.2	
	15	Sulphetrone-100	0	53	78	8.6	1.4
	15	Sulphetrone-500	0	66	10	9.9	1.2
	15	0	5	27	108	6.5	1.9
	15	0	25	7	204	2.0	6.1
	15	0	50	7	188	0.8	15.2
	15	Sulphetrone-100	5	0	175	3.6	3.4
	15	Sulphetrone-500	5	7	184	3.6	3.4
	15	Sulphetrone-100	25	0	240	1.3	9.4
	15	Sulphetrone-500	25	0	248	0.7	17.4

* Average tuberculous involvement for the group, each animal being graded 0 to 20 according to the extent of lesions in (a) omentum and mesenteric glands (b) spleen (c) liver (d) peritoneum, kidneys and testes (e) lungs and mediastinal glands, each graded 0 to 4 on the basis of no visible lesions, slight, moderate, generalized, or extensive.

† Average TB index of controls/treated.

Chemotherapeutic activity: This was studied in experimental pneumococcus infection in mice and experimental tuberculosis in guinea pigs.

Brownlee and associates (4) state that Sulphetrone was inactive in experimental pneumococcus infection in mice. In our experience with monosubstituted derivatives of DDS, such compounds have usually shown considerable activity, frequently comparable with that of DDS, and because of their lower toxicity such compounds have often shown a more favorable chemotherapeutic index (9). In the case of the hydroxyethyl compound 50 per cent survival of

infected mice[8] was obtained with a total dose of 3.0 Gm. per Kg. orally and 2.0 Gm. per Kg. subcutaneously, suspended in olive oil, when given in 8 divided doses over a period of 4 days. In normal mice, a single dose of 8.0 Gm. per Kg. orally and 4.0 Gm. per Kg. subcutaneously failed to kill any animals. In the case of the parent substance DDS, approximately 0.5 Gm. per Kg. protected 50 per cent of infected mice when given in 8 divided doses over a period of 4 days, but the single dose of 0.25 Gm. per Kg. killed about 50 per cent of normal mice, whether given orally or subcutaneously. Thus the chemotherapeutic ratio of hydroxyethyl (LD_{50}/SD_{50}) is more than 4 to 6 times greater than that of DDS. These data are shown in table 8.

The results obtained in experimental tuberculosis in guinea pigs are summarized in table 9. These data concern the activities of Sulphetrone and hydroxyethyl when used alone or in combination with streptomycin. The hydroxyethyl compound was used at the relatively low dose levels of 100 and 300 mg. per Kg. because of the limited supplies at the time this work was done. The Sulphetrone experiments were so planned as to give some information on the question as to which is the more critical, streptomycin or the supplementary sulfone, in combined therapy. The results indicate greater activity for hydroxyethyl than Sulphetrone when used alone, and potentiation of action in the case of the hydroxyethyl compound, comparable with that reported previously for Promin and other sulfones (3, 10). An additive effect in the instance of Sulphetrone was obtained when used with the small dose of 5 mg. per Kg. per day of streptomycin. When used with the larger dose of streptomycin, 25 mg. per Kg. per day, some potentiation of action was obtained. The chemotherapeutic effectiveness of 25 mg. per Kg. streptomycin plus 500 mg. per Kg. Sulphetrone was somewhat greater than that of double the dose of streptomycin when used alone.

SUMMARY AND CONCLUSIONS

A comparative study is presented of the pharmacologic and chemotherapeutic properties of (*1*) 4,4′-diaminodiphenylsulfone (DDS), (*2*) Promin, (*3*) Sulphetrone and (*4*) 4-amino-4′-β-hydroxyethylaminodiphenylsulfone (hydroxyethyl).

The intravenous toxicity of Promin and Sulphetrone in rats is almost identical if computed on the basis of DDS equivalent.

The oral toxicity of Sulphetrone and hydroxyethyl is much less than that of Promin. The low toxicity of Sulphetrone appears to be due to poor absorbability.

The blood levels following oral administration of hydroxyethyl are relatively low. This is not due to poor absorbability but rather to a preferential localization of the compound in certain organs and tissues of the body, *e.g.*, liver, kidney, lungs, and spleen. About 50 per cent of the dose administered is excreted in the urine in 24 hours, and an undetermined but appreciable amount, possibly 25 per cent, is eliminated in the bile.

[8] Mice of 18 to 22 Gm. were used and the infecting dose was 0.5 cc. of 10^{-6} dilution of a six hour broth culture of type I pneumococcus given intraperitoneally, which kills all controls within 24 hours (9).

There is evidence to indicate that Promin and Sulphetrone are metabolized in the body to the parent substance DDS; there is evidence that hydroxyethyl is not metabolized to DDS.

Hydroxyethyl has a good chemotherapeutic activity in experimental pneumococcus infection in mice. Its activity in exerimental tuberculosis in guinea pigs compares favorably with Promin. Like Promin it potentiates the action of streptomycin. Sulphetrone has a lower activity when used alone and, when used with 5 mg. per Kg. per day of streptomycin, the effect was additive. When used with the large dose of 25 mg. per Kg. per day of streptomycin, some potentiation of action was obtained.

SUMARIO Y CONCLUSIONES

La Acción Farmacológica y Quimioterapéutica de Algunas Nuevas Sulfonas y de la Estreptomicina en la Tuberculosis Experimental

Este estudio comparativo versa sobre las propiedades farmacológicas y quimioterapéuticas de: (1) la 4,4′-diaminodifenilsulfona (DDS), (2) la promina, (3) la sulfetrona, y (4) la 4-amino-4′-β-hidroxi-etilaminodifenilsulfona (hidroxietilo).

La toxicidad endovenosa de la promina y la sulfetrona en las ratas es casi idéntica si se computa a base de equivalencia en DDS.

La toxicidad por vía oral de la sulfetrona y el hidroxietilo es mucho menor que la de la promina. La poca toxicidad de la sulfetrona parece deberse a mala absorbibilidad.

Los tenores sanguíneos consecutivos a la administración oral del hidroxietilo son relativamente bajos, lo cual no se debe a mala absorbibilidad sino más bien a localización preferente del compuesto en ciertos órganos y tejidos, por ej., hígado, riñones, pulmones y bazo. Aproximadamente 50 por ciento de la dosis administrada se excreta en la orina en veinticuatro horas y una cantidad indeterminada, pero apreciable, posiblemente 25 por ciento, es eliminada en la bilis.

Hay datos indicativos de que, en el cuerpo, la promina y la sulfetrona se metabolizan en la sustancia matriz, DDS, habiendo también datos de que no sucede otro tanto con el hidroxietilo.

El hidroxietilo muestra buena actividad quimioterpaéutica en la infección neumocócica experimental en los ratones. Su actividad en la tuberculosis experimental del cobayo compárase favorablemente con la de la promina, y lo mismo que ésta, realza la acción de la estreptomicina. La sulfetrona muestra menos actividad cuando se usa por sí sola, y usada con 5 mg. diarios de estreptomicina por kg., mostró efecto aditivo. Usada con una dosis masiva de 25 mg. diarios de estreptomicina por kg., obtuvo alguna potenciación del efecto.

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NEOMYCIN ACTIVITY UPON MYCOBACTERIUM TUBERCULOSIS AND OTHER MYCOBACTERIA[1, 2, 3]

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INTRODUCTION

Before a new antimicrobial agent can be considered at the present time as having chemotherapeutic potentialities, it must possess the following properties:

1. It must be active, not only *in vitro* but also *in vivo*, against certain specific organisms; it is desirable that such activity be greater than that of other agents now recognized as useful or as promising.

2. It must be active against those strains of important disease-producing organisms which are not sensitive or which have become resistant to presently accepted chemotherapeutic agents.

3. It should not favor the development of resistance among sensitive bacteria, or should at least permit lesser development of such resistance than existing agents, or it should act synergistically with those agents.

4. It should not be toxic to animals, or at least it should be no more toxic than agents now used for chemotherapeutic purposes.

These four postulates can be enlarged and others added, as regards both the substances that possess antimicrobial properties, whether antibiotics or synthetic compounds, and the organisms which are thus affected, whether bacteria, fungi, protozoa, or viruses.

During the last five years, or since the isolation of streptomycin in this laboratory, several new agents were obtained that at first appeared promising but which were later discarded because they did not meet the above requirements. This is true, for example, of grisein (1) and streptothricin VI (2). The same is true of other agents isolated in different laboratories.

Because of its marked antimycobacterial properties, streptomycin has so far proved to be the most promising antibiotic in the treatment of infections caused by *Mycobacterium tuberculosis*. It possesses certain serious limitations, however, notably, first, the ease with which sensitive bacteria develop resistance to it and, second, certain toxic manifestations. It becomes, therefore, essential to find new agents which would be less favorable to the development of resistance among bacteria, less toxic, and possibly more potent than streptomycin.

The isolation of such an agent, designated as *neomycin*, recently was announced (3, 4).

On the basis of extensive laboratory studies against various bacteria, including

[1] Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Microbiology.

[2] Supported by a grant from the Rutgers Research and Endowment Foundation.

[3] Some of the data reported in this paper were presented before the seventh Streptomycin Conference, Veterans Administration, held in Denver, Colorado, on April 23, 1949.

both saprophytic and pathogenic mycobacteria, as well as of experimental studies in animals against various gram-positive and gram-negative bacteria (exclusive of *M. tuberculosis*), neomycin appears to meet the above requirements. (*1*) It is more active than streptomycin against both the saprophytic *Mycobacterium* 607 and pathogenic strains of *M. tuberculosis*. (*2*) It is as active against the streptomycin-resistant strains of these organisms as against the sensitive ones. (*3*) It favors to a far lesser degree than does streptomycin the development of resistant strains of mycobacteria although, on prolonged serial transfer of large numbers of cells, a considerable increase in resistance may be obtained. (*4*) It is characterized by a relatively low toxicity to animals.

The degree of toxicity of neomycin, in comparison with that of streptomycin, has not yet been fully established, nor has its *in vivo* activity against the tuberculosis organism.

TABLE 1

Comparative Effects of Neomycin and Streptomycin upon the Growth of Different Mycobacteria

Growth inhibition in units per ml. after 14 days' incubation

TEST ORGANISM	NEOMYCIN	STREPTOMYCIN
M. avium	0.1-0.3	10.0
M. tuberculosis H37 Rv	0.2-1.0	1.0-5.0
M. tuberculosis H37 RvR*	0.2-1.0	>5,000
Mycobacterium 607	0.1	0.2-0.4
Mycobacterium 607R*	0.25	>300

* R = streptomycin-resistant strain.

The results of the following experiments tend to substantiate the foregoing conclusions.

EXPERIMENTAL

Antimycobacterial properties of neomycin: The relatively high potency of neomycin against mycobacteria may be seen in table 1. Different organisms belonging to this group vary greatly, however, in their sensitivity to this antibiotic, the saprophytic *Mycobacterium* 607 being the most sensitive. When pathogenic strains of *M. tuberculosis* were tested in several laboratories, somewhat different results were obtained. This is quite natural, since one must allow not only for the origin of the culture, its past history and method of testing, but also the individual idiosyncrasy of the observer. In general, the various cultures were more sensitive to neomycin than to streptomycin. Only seldom was a streptomycin-resistant culture of *M. tuberculosis* found to be somewhat more resistant to neomycin than the corresponding streptomycin-sensitive culture. Such differences were only minor in nature, however, and may have resulted from variations in procedure. Freshly isolated cultures of *M. tuberculosis* were also found to be more sensitive to neomycin than to streptomycin (table 2). This was true especially of the streptomycin-resistant strains which

showed in this case no difference in sensitivity to neomycin as compared to the streptomycin-sensitive cultures.

When the antimycobacterial properties of neomycin were compared further with those of streptomycin and of streptothricin against both saprophytic and pathogenic strains, certain very striking differences were obtained (table 3).

TABLE 2

Comparative Effects of Neomycin and Streptomycin upon Freshly Isolated Strains of M. tuberculosis

(F. Heilman and G. M. Needham)

STRAIN NUMBER	SOURCE OF CULTURE	AMOUNT INHIBITING GROWTH*	
		Streptomycin	Neomycin
		γ per ml.	*units per ml.*
100	Sinus left hip	1 to 5†	<0.5
500	Sinus left flank	1 to 5	<0.5
575	Bladder	>100	<0.5
910	Gastric	1 to 5	<0.5
209	Sputum	1 to 5	<0.5
684	Urine	>100	<0.5

* Tests made in liquid Proskauer and Beck's medium fortified with 0.25 per cent bovine albumin (fraction V).

† 1 to 5 = more than 1 and less than 5 γ required to inhibit growth; >100 = growth not inhibited by 100 γ.

TABLE 3

Influence of Continuous Incubation upon the Antimycobacterial Properties of Different Antibiotics

ORGANISMS	UNITS OF ANTIBIOTIC REQUIRED TO INHIBIT GROWTH PER MILLILITER OF CULTURE IN DUBOS-TWEEN MEDIUM*					
	Neomycin		Streptomycin		Streptothricin	
	Days of incubation					
	7	14	7	14	7	14
Mycobacterium 607	0.1	0.1	0.3	0.3–0.6	0.75	2
Mycobacterium 607R	0.1–0.25	0.25	200	300	1.0	2
	14	28	14	28	14	28
M. tuberculosis H37 Rv	0.5–1.0	1.5–2.0	2	4	10	20
M. tuberculosis H37 RvR	1.0	1.0	>5,000	>5,000	10	20

* Dilution of inoculum = 1:1,000.

Neomycin was found to possess far superior properties as an antituberculous agent than the other two antibiotics. This expressed itself in at least three distinct ways: (a) neomycin was the most active of the three antibiotics when tested against both saprophytic and pathogenic mycobacteria; (b) neomycin was as active upon the streptomycin-resistant as upon the streptomycin-sensitive

mycobacteria; (c) upon continued incubation of the cultures in the presence of the three antibiotics, the tendency toward the development of resistance to neomycin was less than to the other two antibiotics.

A more detailed study has now been made of the course of growth of both streptomycin-sensitive and streptomycin-resistant strains of *M. tuberculosis* H37 Rv in the presence of three different antibiotics, in order to establish the course

TABLE 4

Comparative Tuberculostatic Effects of Neomycin, Streptomycin and Aureomycin upon the Growth of M. tuberculosis H37 Rv

Turbidimetric readings in Dubos-Tween medium

UNITS PER ML.	NEOMYCIN, INCUBATION IN DAYS				γ PER ML.	STREPTOMYCIN, INCUBATION IN DAYS				γ PER ML.	AUREOMYCIN, INCUBATION IN DAYS		
	4	9	16	28		4	7	14	28		4	7	14
0	15	23	78	225	0	10	21	96	268	0	9	17	40
0.1	0	15	79	244	0.1	5	25	101	256	0.5	7	17	96
0.5	0	0	38	155									
1.0	0	0	11	120	1.0	0	0	41	191	5.0	6	9	64
2.0	0	0	0	0	2.0	0	0	0	37	10.0	0	6	22
					4.0	0	0	0	0	50.0	0	0	0

TABLE 5

Comparative Tuberculostatic Effect of Different Antibiotics upon the Growth of Mycobacterium 607

Turbidimetric readings in Dubos-Tween medium

UNITS PER ML.	NEOMYCIN, INCUBATION IN DAYS			γ PER ML.	STREPTOMYCIN, INCUBATION IN DAYS		
	2	4	16		2	4	14
0	35	80	288	0	24	65	282
0.1	0	0	0	0.1	20	47	185
0.25	0	0	0	0.3	0	0	180
0.5	0	0	0	0.5	0	0	0
UNITS PER ML.	STREPTOTHRICIN, INCUBATION IN DAYS			γ PER ML.	AUREOMYCIN, INCUBATION IN DAYS		
	2	4	14		2	4	14
0	28	98	270	0	48	82	245
0.1	15	65	260	0.5	33	75	248
0.5	0	5	180	5.0	0	12	216
1.0	0	0	55	10.0	0	0	160

of development of resistance. Only the growth of the sensitive strains is reported in table 4. The amount of neomycin required to inhibit completely the growth of both strains of this organism was 1.0 to 2.0 units per ml., after a prolonged incubation period. Although even smaller concentrations of the antibiotic were found to cause partial inhibition of growth, this effect was later overcome, either because of the gradual adaptation of the culture or because of

development of the more resistant cells. Beyond the concentration of 2 units per ml., however, no growth appeared even after four weeks' incubation of cultures. Streptomycin allowed the more rapid adaption of the organism to the antibiotic, and the final inhibiting concentration was about twice as high as for neomycin. The third antibiotic used in this experiment, aureomycin, showed up rather poorly in comparison with the other two.

Similar results were obtained when the action of several antibiotics against the saprophytic culture *Mycobacterium* 607 was measured (table 5). The growth of this organism was completely inhibited by 0.1 unit per ml. of neomycin; no resistance developed even after prolonged incubation. There wa comparatively little difference in the effect of neomycin upon the streptomycin-sensitive and streptomycin-resistant strains, the latter showing some growth at 0.1 unit per ml. after prolonged incubation of the culture, but not above that concentration. Streptomycin was only about one-third as active upon the saprophytic mycobacterium as neomycin, the activity of streptothricin being less than one-tenth, and of aureomycin less than one-hundredth.

In further studies of the effect of neomycin upon the growth of mycobacteria, it was found that the size of inoculum was of considerable importance within certain limits. When an inoculum of 1:10 was used, 0.2 unit per ml. of neomycin was required to inhibit the growth of *Mycobacterium* 607; when the inoculum was 1:1,000, growth was completely inhibited by 0.1 unit per ml. When repeated transfers, using approximately 122,000 cells, to fresh medium were made from those cultures in which growth occurred in presence of neomycin, only a very limited increase in resistance took place. These results emphasize further the fact that neomycin favors much less the development of resistance among mycobacteria than any of the other antibiotics tested.

A series of plates containing varying concentrations of neomycin were inoculated with large numbers of viable cells of *Mycobacterium* 607 (2 million cells per plate). Only 0.1 unit per ml. permitted growth. When pieces of agar were removed after seven days' incubation and placed in fresh liquid medium, growth occurred from the plates containing 0.1 unit per ml. after two days, 0.5 unit per ml. to 1.0 unit per ml. after six days. When such pieces of agar were removed after fourteen days' incubation and placed in fresh liquid medium, growth occurred only from the 0.1 unit per ml. plates, but not from those containing the higher concentrations of the antibiotic. This points to the marked bactericidal effects of neomycin. These observations were confirmed in various subsequent quantitative studies. In a more detailed study, the mycobactericidal properties of neomycin, streptomycin, and streptothricin were compared. Washed suspensions of mycobacteria were used and the number of cells was measured by the plate method. It was found that neomycin was the most bactericidal of the three antibiotics and streptothricin the least.

Comparative effects of streptomycin and neomycin upon Mycobacterium 607: Although the saprophytic *Mycobacterium* 607 behaves in its physiology, rate of growth, and other properties in a distinctly different manner from the pathogenic mycobacteria, it lends itself readily to detailed studies of the type planned here.

Whenever possible, such experiments were finally checked by the use of pathogenic forms.

It has been shown that *Mycobacterium* 607 is sensitive to 0.1 unit of neomycin. This varies somewhat depending on the medium and the length of incubation period. In conformity with the above results, it was found that this organism is not only more sensitive to neomycin than to streptomycin, but that it also allows less development of resistant strains, as may be seen in figure 1. The ratio of sensitivity of *Mycobacterium* 607 to streptomycin, as compared to neomycin, is 0.4:0.1, the latter antibiotic being thus four times as effective as streptomycin against this organism.

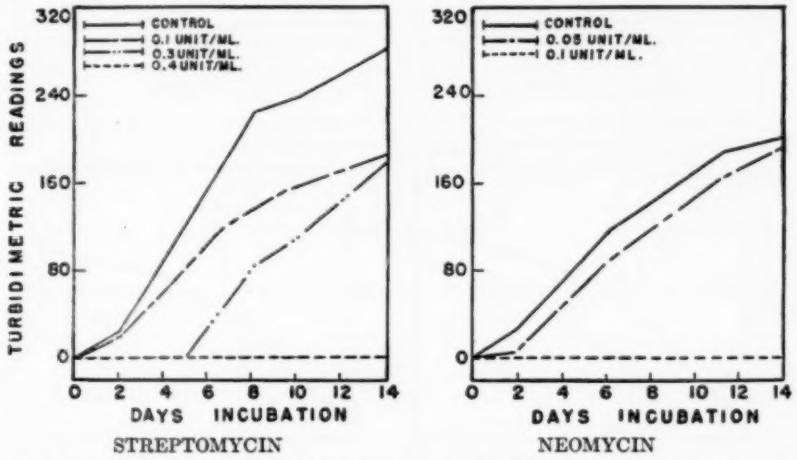

FIG. 1. Inhibition of growth of *Mycobacterium* 607 by different concentrations of streptomycin and neomycin.

That neomycin is as effective upon the streptomycin-resistant strain of *Mycobacterium* 607, namely 607R, as upon the sensitive one is illustrated in figure 2. The culture used in this experiment was not made absolutely resistant to streptomycin, since it was usually inhibited by 400 γ per ml. of this antibiotic; as compared to the sensitivity of the normal strain (0.4 γ per ml.), it may be considered relatively resistant. The resistance of this strain to neomycin is only slightly greater, namely 0.2 unit per ml. as compared to 0.1 unit per ml. in the case of the streptomycin-sensitive strain.

Effect of inoculum upon the sensitivity of Mycobacterim 607 to neomycin: It was pointed out above that neomycin does not favor the rapid development of resistant strains. In these studies, a 7 day old culture of the organism was diluted one thousand fold with Dubos-Tween medium, and turbidimetric readings were made daily with a Klett-Summerson photolometer. To test the effect of inoculum upon the activity of neomycin, a 5 day old culture of *Mycobacterium*

607 was diluted with the Dubos-Tween medium to 1:10, 1:100, 1:1,000. Duplicate tubes were incubated and readings made daily. The results (figure 3)

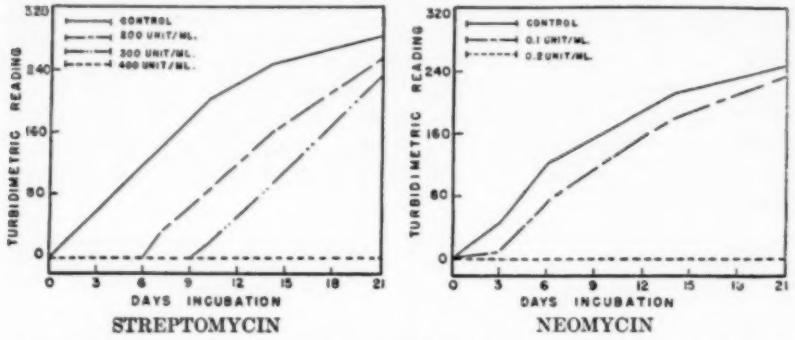

FIG. 2. Inhibition of growth of streptomycin-resistant strain of *Mycobacterium* 607 (607R) by different concentrations of streptomycin and neomycin.

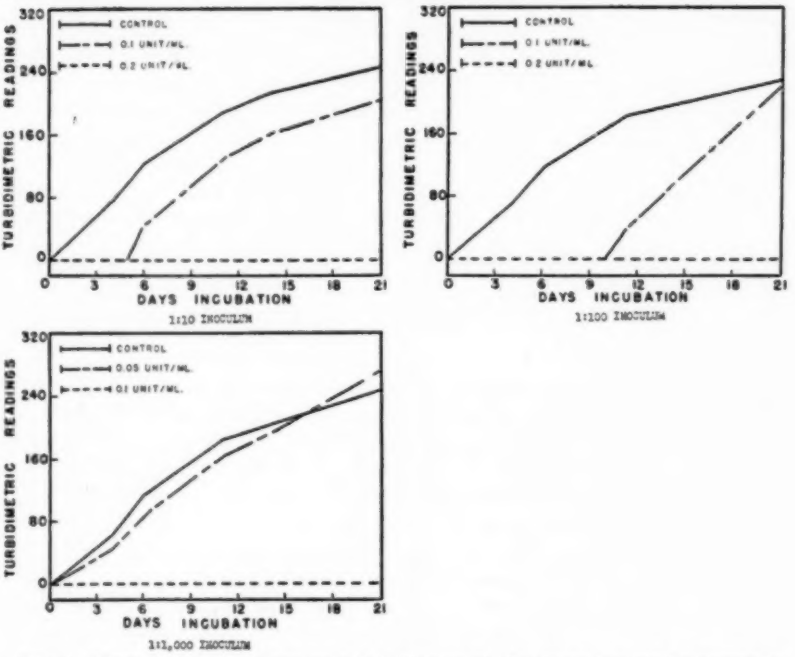

FIG. 3. Effect of inoculum upon growth inhibition of *Mycobacterium* 607 by neomycin.

show that in the 1:1,000 inoculum the limiting effective concentration of neomycin is 0.1 unit per ml., whereas the 1:10 inoculum requires 0.2 unit per ml. for growth inhibition. The 1:100 inoculum fell between.

When repeated transfers were made from the tubes giving growth of the organism in the presence of 0.05 unit per ml. and 0.1 unit per ml. of neomycin into fresh tubes containing varying concentrations of the antibiotic, no marked

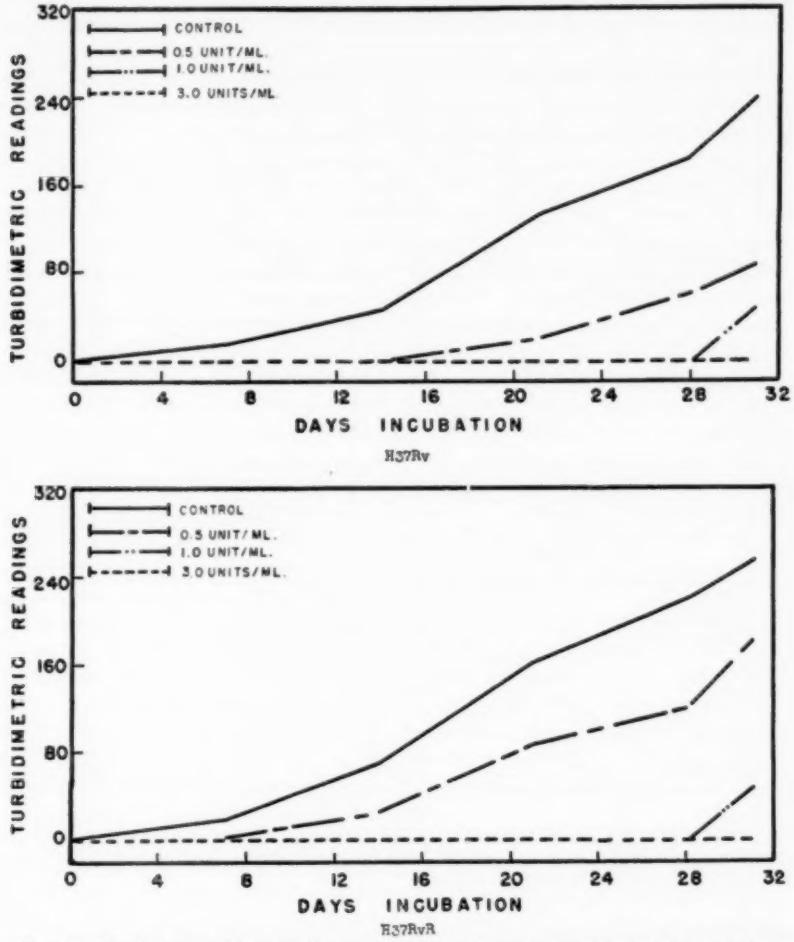

FIG. 4. Effect of neomycin upon the growth of streptomycin-sensitive strains and streptomycin-resistant strains of *Mycobacterium tuberculosis*.

increase in resistance was obtained. The slight increase frequently observed usually expressed itself in an earlier initiation of growth at a given limiting zone of inhibition, *i.e.*, growth in presence of 0.1 unit per ml. was now observed first after one to three days' incubation as compared to the previous six to eleven days. The 1:1,000 dilution of the culture which was grown in the presence of neomycin

required 0.2 unit per ml. for inhibition of growth in place of the original 0.1 unit per ml. In general, when large numbers of *Mycobacterium* 607 were placed in contact with neomycin and with streptomycin in glycerol-nutrient agar media, neomycin proved to be much more bacteriostatic than streptomycin. In one experiment 0.5 unit per ml. of neomycin completely inhibited the growth of

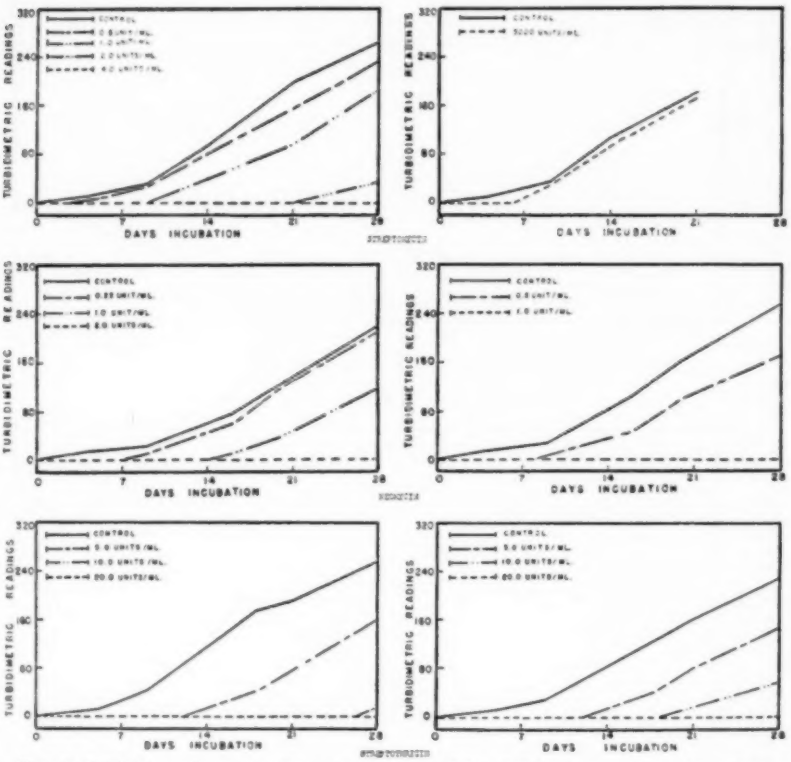

FIG. 5. Inhibition of growth of streptomycin-sensitive (left) and streptomycin-resistant (right) strains of *M. tuberculosis* H37 Rv by 3 basic antibiotics: upper—streptomycin; middle—neomycin; lower—streptothricin.

36,300,000 cells, whereas 1.0 γ per ml. of streptomycin did not inhibit one-hundredth that number of cells.

Effect of neomycin and other antibiotics upon the growth of M. tuberculosis H37 Rv: The amount of neomycin required to inhibit growth of the pathogenic *M. tuberculosis* H37 Rv and of the streptomycin-resistant H37 RvR is somewhat greater than that necessary to inhibit growth of the saprophytes. This amount was reported from different laboratories to vary from 0.2 unit per ml. to 2.0 units per ml. Here again, the incubation period was found to be very important, as

may be seen in figure 4. After fourteen days' incubation, 0.5 unit per ml. was found to suffice for inhibition of growth; after twenty-eight days, however, one unit was required; with longer periods of incubation, even higher concentrations may become necessary. This is true of both the streptomycin-sensitive and streptomycin-resistant strains.

Comparison of the effect of neomycin upon *M. tuberculosis* H37 Rv with that of streptomycin and streptothricin (figure 5) brings out several important points which can be summarized as follows: (a) neomycin is at least equally effective upon the streptomycin-resistant as upon the streptomycin-sensitive strain; (b) neomycin is about twice as active upon *M. tuberculosis* as is streptomycin; (c) streptothricin is the least effective of the three antibiotics. Although streptothricin is also effective upon the streptomycin-resistant strains, nearly ten times as many units or more are required, as compared to neomycin, to bring about the same degree of inhibition. These results can lead to only one conclusion, namely, that of the three basic antibiotics active upon *M. tuberculosis*, neomycin is the most potent and streptothricin the least.

For the sake of completeness, it may be of interest to record here certain pertinent data on the effects upon mycobacteria of another antibiotic produced by a member of the genus *Streptomyces*, namely aureomycin, as shown in some of the tables reported previously. Whereas streptomycin inhibited the growth of *Mycobacterium* 607 for four days when the concentration was 0.2 γ per ml. and for fourteen days when 0.4 γ per ml., it took 5 γ per ml. or 25 times as much aureomycin to produce the same inhibition in two days, and 50 γ per ml. or 125 times as much to inhibit the growth of the organism for fourteen days. Aureomycin was found to be effective alike against the streptomycin-resistant and the streptomycin-sensitive strains of *Mycobacterium* 607.

The growth of *M. tuberculosis* H37 Rv was inhibited by 1 γ per ml. of streptomycin during seven days; 2 γ per ml. were required for growth inhibition in twenty-one days, and 4 γ per ml. for complete inhibition during twenty-eight days of incubation. In the case of aureomycin, however, 10 γ per ml. were required to inhibit growth of the organism in four days and 50 γ per ml. for growth inhibition in seven days, or 10 to 50 times the amount of streptomycin. Because of these limited effects *in vitro*, aureomycin does not appear to be an antituberculous agent of promise.

Toxicity and in vivo activity of neomycin: Treatment of experimental tuberculosis requires larger amounts and more repeated administration than treatment of ordinary infections caused by gram-negative and gram-positive bacteria. In the case of the latter organisms, including such forms as *Staphylococcus aureus*, *Salmonella schottmülleri*, and *Salmonella pullorum*, it was found that a single dose of 100 units by subcutaneous administration in mice or upon the allantoic membrane in chick embryos was sufficient to bring about protection. Frequently, a much smaller dose was effective. The preparations used for this purpose showed a toxic level of 2,000 to 5,000 units per 18 to 20 Gm. mouse, or a ratio of 20 to 50 between toxicity and therapeutic activity.

In an effort to produce larger amounts of neomycin, especially in changing the

composition of the medium to obtain higher potency material, certain toxic preparations were encountered. This complicated somewhat the effort made to obtain immediate information concerning its *in vivo* antituberculous activity. Before the product is standardized, before the toxic contaminations have been eliminated, before suitable dosages have been worked out, it is difficult to say how this new antibiotic will fit into the therapy of tuberculosis.

SUMMARY

The results of a comparative study of the antimycobacterial properties of a new antibiotic, neomycin, and certain other antibiotics, including streptomycin, are presented. These results permit the following conclusions:

1. Neomycin is more active than other antibiotics against both pathogenic and saprophytic mycobacteria.

2. Neomycin is just as active against the streptomycin-sensitive as against the streptomycin-resistant mycobacteria.

3. Neomycin does not allow a rapid development of resistance among the mycobacteria as does streptomycin. Repeated transfers of the organism to media containing neomycin brought about only a slight increase in resistance.

4. The amount of neomycin required to inhibit the growth of mycobacteria depends upon nature of organism, size of inoculum, and length of incubation period. The saprophytic *Mycobacterium* 607 is inhibited by about 0.1 unit per ml. and the pathogenic *M. tuberculosis* by 0.2 to 1.0 unit per ml.

5. Results of the *in vivo* studies against tuberculosis in experimental animals are still insufficient to justify broad conclusions. Neomycin is highly effective against the ordinary pathogenic gram-negative and gram-positive bacteria, the activity dose being one-twentieth to one-fiftieth that of the toxic dose. This is true for both streptomycin-sensitive and streptomycin-resistant organisms. The evidence further suggests that neomycin is also more active against those bacteria than is streptomycin.

SUMARIO

Acción de la Neomicina sobre el Mycobacterium tuberculosis y Otras Micobacterias

Los resultados expuestos derívanse de un estudio comparativo de las propiedades antimicobacterianas de un nuevo antibiótico, la neomicina, y de ciertos otros antibióticos, incluso la estreptomicina, cabiendo sacar las siguientes conclusiones:

1. La neomicina es más activa que otros antibióticos contra las micobacterias, tanto patógenas cuanto saprofitas.

2. La neomicina es igualmente activa contra las micobacterias tanto estreptomicino-sensibles cuanto resistentes.

3. La neomicina no permite, como la estreptomicina, rápida formación de resistencia en las micobacterias. Los pases repetidos de los microbios a medios que contenían neomicina sólo consiguieron leve aumento de la resistencia.

4. La cantidad de neomicina necesaria para inhibir el desarrollo de las mico-

bacterias depende de la naturaleza del microbio, la cantidad de inóculo y la duración del período de incubación. El saprofito *Mycobacterium* 607 is inhibido aproximadamente por 0.1 unidad por ml. y el patógeno *M. tuberculosis* por 0.2 a 1.0 unidad por ml.

5. Los resultados de los estudios *in vivo* contra la tuberculosis en los animales de experimentación son todavía insuficientes para sacar amplias conclusiones. La neomicina es altamente eficaz contra las ordinarias bacterias patógenas gram-negativas y positivas, siendo la dosis activa de una vigésima a una centésima de la tóxica, lo cual reza tanto con los microbios estreptomicino-sensibles como con los resistentes. Los datos disponibles indican además que la neomicina, por vía oral, es también más activa que la estreptomicina contra dichas bacterias.

Acknowledgment

The authors wish to express their sincere appreciation to Dr. F. Heilman of the Mayo Clinic for permission to use the data incorporated in table 2.

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THE USE OF THE MOUSE IN A STANDARDIZED TEST FOR ANTITUBERCULOUS ACTIVITY OF COMPOUNDS OF NATURAL OR SYNTHETIC ORIGIN[1]

I. Choice and Standardization of Culture

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INTRODUCTION

As dramatic successes have followed one another in the discovery of substances curative in most acute and some chronic bacterial infections, attention has been focused on those infections where such success has not been so complete. One such infection is tuberculosis. Although the discovery of streptomycin (1) has provided a drug of the greatest importance, most investigators and clinicians feel that it, or it alone, is not the final word in the therapy of this infection.

In the investigation of large series of substances for action *in vivo* against the tubercle bacillus, the use of a satisfactory experimental animal is of prime importance. In the past the animal of choice has been the guinea pig, although the rabbit and the mouse have also been used. Some investigators, indeed, believe that the results of such *in vivo* tests are only valid for man if carried out in the highly suceptible guinea pig. The present writers have been led to abandon this latter animal in favor of the mouse for the following reasons. Work with mice in tuberculosis and other infections has shown the importance of the use of homozygous animals when few animals can be tested and when, for this and other reasons, uniformity of response is desirable. No ready source of homozygous guinea pigs is available while, on the other hand, many homozygous strains of mice are readily available. Since many of the drugs to be tested are in short supply, it is of the utmost importance to use a small animal, and in the fewest numbers which will give reproducible results. As will appear in the third paper of this series (2), using homozygous mice in groups of 10 per dose level, chronic toxicity tests and tests of therapeutic effectiveness, as well as *in vitro* tests, can be carried out with 20 grams or less of material. Furthermore, the use of the mouse entails a far lower immediate and continuing expense than does the use of a larger animal, since the initial cost is lower and mice can subsequently be housed and fed at appreciably lower cost than can guinea pigs. Finally, there is no evidence that the results of chemotherapy in tuberculosis of the guinea pig are a more certain guide to the usefulness of a given compound in man than are the results obtained in mice. Indeed the reverse appears actually to be the case.

A consideration of these facts has led us to an investigation of the use of the

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mouse for *in vivo* tests for chemotherapy of tuberculosis. The standardization and use of such a test will form the material for the subsequent series of reports.

LITERATURE

Although the first observation on the use of the mouse in experimental tuberculosis was made in 1884 by Koch (3), this animal has been, until recently, very little used. Since Raleigh and Youmans (4) have published a comprehensive review of the literature, mention will be made here only of such work as deals specifically with the relative virulence of *M. tuberculosis* var. hominis, var. bovis, and *mycobacterium avium*.

Among the first to investigate the relative virulence for mice of the different types of *M. tuberculosis* was Römer (5) in 1903, who compared the virulence of 4 human and 5 bovine strains. Since only one of the human strains approached the bovine in virulence, he concluded that bovine strains were more virulent. Infection was more readily produced by intraperitoneal than by subcutaneous injection.

In the studies of the Royal Commission on Tuberculosis (6), although extensive experiments with many species of animals were carried out, very little work was done with the mouse. The two routes of injection used in the mouse experiments were the subcutaneous and intraperitoneal. In the few mice used, the dosage varied so greatly that no conclusion could be reached as to the relative virulence of human, bovine, and avian strains of *M. tuberculosis* for this animal.

Trommsdorff (7) in 1909 tested several bovine and human strains of *M. tuberculosis* by intravenous injection into mice and found the bovine strains more virulent. Peters (8) in 1912 injected mice intravenously with 1 mg. doses of 2 human and 2 bovine strains. Only 3 to 4 mice were used for each strain. Mice injected with the bovine strains died between 4 and 7 weeks while mice injected with the human strains showed relatively slight lesions in the organs when killed 8 to 9 weeks after injection. On the basis of these meager data, he concluded that the mouse could be used to differentiate human and bovine strains. Binder (9) injected 1 mg. doses of one human and one bovine strain intravenously into white mice. Six mice injected with the bovine strain died between the twenty-seventh and fifty-seventh days, while 7 mice injected with the human strain showed only a few discrete lesions when killed between the one hundred thirtieth and one hundred eighty-eighth days. The results of Trommsdorff, Peters and Binder, although obtained with small numbers of mice, are in agreement. On the other hand, Lange (10) in 1922, using 4 human and 3 bovine strains, injected mice intravenously with doses of 1.0, 0.1, 0.01, and 0.001 mg. and concluded that the virulence of the two types was the same. In this work also, the number of mice used was small, only 2 to 6 mice for each dose of culture.

Browning and Gulbransen (11) in 1926 tested the virulence for mice of 7 human and 6 bovine strains. Three mice only were used for each culture. The dose in all cases was 0.75 mg. injected intraperitoneally. In general, mice infected with the human strains lived longer than those infected with the bovine strains. Also, several human strains failed to produce infection whereas no failure occurred with the bovine strains.

Gunn, Nungester, and Hougen (12) in 1934, using recently isolated human, bovine, and avian strains, found mice equally susceptible to the human and bovine but resistant to avian strains. One mg. doses of human and bovine strains injected intraperitoneally gave average survival times respectively of 46 and 45 days. The average survival time following intravenous injection of smaller doses was much shorter. Avian bacilli never caused a fatal infection even when a large dose (1 mg.) was injected intravenously, but characteristic lesions were produced in the liver and spleen while the lungs were usually free of gross lesions.

Schwabacher and Wilson (13) in 1937 tested the virulence for mice of 2 human, 2 bovine, and 4 avian strains. A dose of 200 million bacilli injected intravenously into 8 to 21 mice was used. The human and bovine strains were found to be equally virulent, all infected mice died, and the mean time of death was 25.5 and 32.3 days for the human strains and 22.8 and 30.9 for the bovine strains. The avian strains were less virulent and showed wide varia-

tion in virulence, approximately one-half the mice survived and the mean times of death for the 4 strains were 39.6, 57.5, 108.5, and 138.3 days.

Stamatin and Stamatin (14) in 1939 carried out an extensive study of the virulence for white mice of recently isolated strains. Ten human strains and 9 variants, 10 bovine strains and 2 variants, and 4 avian strains were used. The cultures were injected intravenously in doses of 1.0, 0.1, 0.01, and 0.001 mg. Results with the human and bovine strains were similar with the larger doses but with the smallest dose (0.001 mg.) the length of life was shorter and the lesions more severe in mice injected with the bovine strains. Avian strains were much less virulent than human or bovine strains.

Pagel (15) in 1940 observed that human and bovine strains when injected intracutaneously into mice induced more pronounced lesions than avian strains. Long and Vogt (16) in 1941 found no difference in the response of white mice to intraperitoneal injection of 1.0 mg. doses of a human and a bovine (Ravenel) strain.

It is somewhat difficult to draw definite conclusions from the data presented by these investigators since no standard procedures were used. While there is disagreement in regard to the relative virulence for mice of human and bovine strains, it is clear that both possess far greater virulence than the avian type. There are undoubtedly strain differences within types but it appears that bovine strains may be universally more virulent than human strains.

In the present series of papers, investigation leading to the establishment of a standardized test in mice for antituberculous activity is outlined. In evolving such a test the first consideration was choice of a satisfactory strain of *M. tuberculosis*. Such a strain must possess sufficiently high and constant virulence to give reproducible results. The choice of culture, dosage, and methods of maintenance are presented in the first paper of this series.

MATERIALS AND METHODS

Cultures: Two strains of *M. tuberculosis* var. hominis, H37 Rv and R, and two strains of var. bovis, Ravenel and D4, were used in the present study. The H37 Rv strain was obtained from Mr. William Steenken, Jr., Trudeau, New York, and the R strain from Dr. Arnold Rich, the Johns Hopkins Hospital, Baltimore, Maryland. The Ravenel strain was obtained from Dr. Max Lurie, Henry Phipps Institute, Philadelphia, Pennsylvania, and the D4 strain from Dr. Harold J. White, American Cyanamid Co., Stamford, Connecticut, who had obtained it from Dr. Maurice L. Cohn, National Jewish Hospital, Denver, Colorado.

Medium: Stock cultures were kept stored at 3°C. on the Jensen and Holmes modification of Loewenstein's medium (17). Because of the difficulty experienced in obtaining a sterile medium, even with inspissation repeated on three successive days, a preliminary sterilization of the potato flour was introduced. When this was done, one inspissation only was sufficient to give a sterile medium. The formula is given below.

Loewenstein Medium

(a) Mineral salt solution

Anhydrous potassium sulphate	1.2	Gm.
Anhydrous magnesium sulphate	0.12	Gm.
Magnesium citrate	0.3	Gm.
Asparagine	1.8	Gm.
Glycerine	6	ml.
Distilled water to make	300	ml.

Dissolve and sterilize at 100°C. for two hours.

(b) Sterilize 15 Gm. potato flour in a 2 liter Erlenmeyer flask in hot air oven at 130°C. for two hours. When cool, add mineral salt solution and place in boiling water bath. Agitate constantly until a soft paste is produced (20 to 25 minutes). Place in 56°C. water bath for one hour.

(c) Wash 10 to 12 fresh eggs and place in 70 per cent alcohol for ten to fifteen minutes. Mix eggs in sterile Waring blender for one-half minute and add to mineral salt starch solution.

(d) Add 10 ml. of a 2 per cent Malachite green solution which has been incubated at 37°C. overnight. Mix thoroughly and filter through sterile gauze. Tube and place in Arnold sterilizer at 50°C. Take temperature up slowly to 75°C. (this should take about one hour). Hold at 75°C. for forty-five minutes. No subsequent sterilization is necessary.

Cultures to be used for mouse injection were grown, after the manner of Dubos (18), in a liquid medium containing Tween 80 and serum albumin. As a basic medium, Kirchner's (19) synthetic medium, to which was added 0.05 per cent Tween 80 to induce diffuse growth and 0.1 per cent human serum albumin, was used. A 25 per cent solution of human serum albumin in distilled water, sterilized by filtration, was added to the previously autoclaved Kirchner-Tween 80 medium in an amount sufficient to make a final concentration of 0.1 per cent serum albumin. The formula for the Kirchner medium is as follows.

Kirchner Synthetic Medium

$NaHPO_4$	3.0 Gm.
KH_2PO_4	4.0 Gm.
$MgSO_4$	0.6 Gm.
Sodium citrate	2.5 Gm.
Asparagine	5.0 Gm.
Iron ammonium citrate	0.05 Gm.
Glycerine	20 ml.
Distilled water	1,000 ml.

Maintenance of cultures: The cultures to be used for mouse injection were grown in 50 ml. Erlenmeyer flasks containing 20 ml. of Kirchner-Tween 80 serum albumin medium. An inoculum of 2 ml. of a culture grown in the same manner was used. Cultures were incubated for five days at 37°C.

Mice: CFl albino mice weighing 16 to 20 Gm. were used in all experiments. The injections were made by the intravenous route. Cultures were diluted so that the required dose was contained in a volume of 0.5 ml. since it was believed that this volume ensured a more constant dose than could be achieved by the use of smaller volumes such as 0.1 or 0.2 ml. Throughout this paper the dosage will be given in terms of undiluted culture, that is, if the dose used is 0.5 ml. of a 1:10 dilution it will be given as 0.05 ml. of undiluted culture. The mice were observed daily and group weights were taken at intervals of three to four days. Autopsies were performed on all mice to exclude the possibility of death from causes other than tuberculosis.

In the experiments which follow, the t_{50}, or calculated 50 per cent survival time, was used in all virulence studies. The 50 per cent survival times were calculated from time-mortality curves according to the method described in the second paper of this series (20).

RESULTS

Choice of Medium

Experiments in vitro: The announcement by Dubos (18) and Dubos and Davis (21) of a medium in which tubercle bacilli grew rapidly and diffusely, and yet retained their acid-fast characteristics and virulence, offered the means of simplifying both the cultivation of tubercle bacilli and the standardization of suspensions to be used in experimental work. The agent which they found to be most satisfactory for the production of diffuse growth was Tween 80 (polyoxyethylene derivative of sorbitan mono-oleate) a non-ionic surface active compound obtained from the Atlas Products Co. of Wilmington, Delaware. It was found that, while Tween 80 induced rapid and diffuse growth of the tubercle bacillus, there was

present in the commercial preparations an amount of unesterified oleic acid sufficient to exert a growth-inhibitory effect. This could be removed chemically from the commercial product (22) or counteracted by the addition of serum albumin to the medium.

Dubos and co-workers (21, 23, 24, 25), after extensive investigations on the composition of media containing Tween 80 and serum albumin, have recommended certain basic formulae. Sattler and Youmans (26), using both a modified Proskauer and Beck synthetic medium and the basic medium of Dubos, have studied the effect on growth of the H37 Rv strain of the addition of Tween 80 and serum albumin to the medium. These investigators found that Tween 80, either purified or unpurified, had an inhibitory effect on growth. When 0.2 per cent serum albumin was added, there was no inhibition for the first five days but thereafter a decrease in growth was noted in the media containing Tween 80. Growth in the modified Proskauer and Beck medium appeared to be somewhat better than in the Dubos basic medium.

In this laboratory no extensive study of the relative merits of various media has been made. However, a comparison was made of Kirchner, Proskauer and Beck, and Long's synthetic media for the support of surface growth of *M. tuberculosis*. Blake bottles containing the three media were inoculated by loop transfer with the H37 Rv and Ravenel strains grown on the surface of Kirchner's medium. Growth of the H37 Rv strain was equally rapid on the Kirchner and Proskauer and Beck media but was slower on Long's medium. At a time when the surface of the two former media was entirely covered by growth, only slight spread had occurred on Long's medium. Growth of the Ravenel strain was more rapid on Kirchner's medium than on the Proskauer and Beck and was very slow on Long's medium.

A conference with Dr. Dubos at this time revealed that he also had found Long's medium less satisfactory than one of his own devising which was much like the Kirchner medium in composition.

A comparison of the growth of 2 human strains of *M. tuberculosis* (H37 Rv and R) and 2 bovine strains (Ravenel and D4) was made in the following media: Kirchner's medium, with and without glycerine, containing 0.05 per cent Tween 80 with 0.05, 0.1, and 0.2 per cent human serum albumin; and Dubos (21) Tween 80 serum albumin medium with asparagine, and the same with enzymatic hydrolysate of casein. The various media were distributed in 150 by 18 mm. Pyrex glass tubes in 5 ml. amounts. Cultures for seeding, which were grown in Kirchner-Tween 80 serum albumin medium, were added to the various media to make final concentrations of 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400, and 1:12,800. The tubes which were incubated at 37°C. were observed at two day intervals and the degree of turbidity noted.

The rapidity of growth of the 4 strains in decreasing order was H37 Rv, R, Ravenel, and D4. The human strains grew equally well in all media. Growth of the Ravenel strain was inhibited or retarded in the Kirchner medium containing the least amount (0.05 per cent) of serum albumin, an amount probably insufficient to neutralize the free oleic acid present in the Tween 80. Growth occurred equally well in all other media. The presence of glycerine in the Kirchner medium exerted a retarding effect on the growth of the D4 strain.

Experiments in vivo: The comparative virulence for mice of the H37 Rv and Ravenel strains, when grown both as a pellicle on the surface of Kirchner's

medium and diffusely in the same medium containing 0.05 per cent Tween 80 and 0.1 per cent human serum albumin, was tested in one experiment.

Suspensions of cultures grown for seventeen days on the surface of Kirchner's medium were prepared as follows: The cultures were drained free of fluid on sterile filter paper, removed from the paper, weighed, ground in a Ten Broeck Pyrex glass tissue grinder, and suspended in normal saline in a concentration of 0.1 mg. per ml. The cultures which were grown in a medium containing Tween 80 and serum albumin were transferred four times in the medium before use in the virulence test in mice. The cultures were diluted to a turbidity corresponding to that of the 0.1 mg. per ml. suspensions. As in general, the H37 Rv strain grew more luxuriantly than the Ravenel, the former was diluted 1:20 and the latter 1:10. All cultures, properly diluted, were injected intravenously in 0.5 ml. amounts into groups of 10 mice each.

It is realized that turbidity determinations give only a crude estimate of dosage. Therefore, as a further means of estimating the uniformity of the cultures, they were diluted and plated on the surface of Loewenstein's medium for colony count. In later work, in which cultures were plated on both Loewenstein's medium and on Kirchner's agar containing Tween 80 and serum albumin, it was found that higher counts were obtained on the Kirchner agar medium. In neither case, however, can it be assumed that the count represents the actual number of organisms viable and infective for the mouse.

In table 1 the results of this experiment on the comparative virulence of surface and diffusely grown cultures are given. From the limited data obtained, it would appear that diffusely grown cultures are at least as virulent for the mouse as those grown on the surface in the traditional manner. This is in confirmation of the work of Dubos and co-workers (23, 27) who have amply demonstrated the virulence of cultures grown diffusely. Because of the demonstrated virulence of diffusely grown cultures and the fact that they yield more rapid growth and are easier and safer to handle and to standardize, this type of culture was used in all further work.

In three different experiments comparison was made of the virulence of the Ravenel culture grown in Kirchner's medium containing 0.05 per cent Tween 80 and 0.1 per cent human serum albumin and in Dubos basal Tween-albumin medium (24). Groups of 10 mice each received intravenous injection of the two cultures standardized to the same turbidity. The dose used (except one culture) was 0.5 ml. of a 1:10 dilution or 0.05 ml. of undiluted culture. In the first experiment, growth of the culture in Dubos medium was heavier than in the Kirchner-Tween serum albumin medium. Consequently, the former culture was diluted 1:15 in order that the cultures as injected should have the same turbidity.

The culture grown in the Kirchner-Tween 80 serum albumin medium proved to be the more virulent. As evidence to be presented later will show, the Ravenel culture contains organisms differing in degree of virulence. It is possible that the Dubos medium might favor the growth of the organisms of lesser virulence. The results are given in table 2.

Since experiments *in vitro* had shown that Kirchner's medium, containing 0.05 per cent Tween 80 and 0.1 per cent human serum albumin, satisfactorily supported growth of cultures of *M. tuberculosis*, and experiments *in vivo* had demonstrated that cultures grown in this medium were virulent for mice, it was adopted as the standard medium.

Choice of Strain of M. tuberculosis

Since the comparative avirulence for mice of avian strains of *M. tuberculosis* has been amply demonstrated (12,13,14), only the 2 human and the 2 bovine strains discussed above were studied for virulence.

TABLE 1

Comparison of Virulence of Surface and Diffuse Growth of the H37 Rv and Ravenel Strains of M. tuberculosis

STRAIN	TYPE OF GROWTH	NUMBER OF MICE	DOSE INTRAVENOUS	NUMBER ORGANISMS INJECTED	T_{50}
H37 Rv	Surface	10	0.05 mg.	186,000	34
H37 Rv	Diffuse	10	0.025 ml.	407,500	25
Ravenel	Surface	10	0.05 mg.	200,000	22
Ravenel	Diffuse	10	0.05 ml.	150,000	18.2

TABLE 2

Comparison of Virulence of M. tuberculosis Ravenel Grown in Kirchner-Tween 80 Medium and in Dubos-Tween 80 Medium

EXPERIMENT	CULTURE GROWN IN KIRCHNER MEDIUM				CULTURE GROWN IN DUBOS MEDIUM			
	Number of mice	Dose	Number organisms injected*	T_{50}	Number of mice	Dose	Number Organisms injected*	T_{50}
		ml.				*ml.*		
1	10	0.05	1,200,000	21.8	10	0.033	1,600,000	34
2	10	0.05		18.0	10	0.05		24
3	10	0.05	400,000	19.0	10	0.05	550,000	23

* These figures were obtained from colony counts made from platings on Loewenstein's medium. The cultures in experiment 2 were not plated.

Cultures to be used for mouse injection were grown for five days at 37°C. in Kirchner's medium containing 0.05 per cent Tween 80 and 0.1 per cent human serum albumin. The cultures, diluted 1:10 in saline, were injected intravenously in 0.5 ml. amount into CF1 albino mice weighing between 16 and 20 Gm. The dose, in terms of undiluted culture, was thus 0.05 ml. The numbers of mice infected with the different cultures were: Ravenel, 150; H37 Rv, 106; D4, 30; and R, 31.

The cumulative rates of death produced by the 4 cultures plotted on arithmetic-probability paper are shown in figure 1.

When the cultures are compared at the time of 50 per cent mortality, they can be arranged in decreasing order of virulence as follows: Ravenel, D4,

H37 Rv, and R. This represents a difference in time of death between the first and second and the second and third cultures each of only about 1½ days and between the last two of about 13 days. The R strain was thus shown to be much less virulent than the other three strains. When the Ravenel, H37 Rv, and D4 cultures are compared at the time of 80 per cent mortality, the greater virulence of the Ravenel culture becomes more apparent. At this time, the cultures arranged in decreasing order of virulence are Ravenel, H37 Rv, and D4, with approximately 5 and 8 days difference respectively between he Ravenel and the two cultures of lesser virulence, H37 Rv and D4.

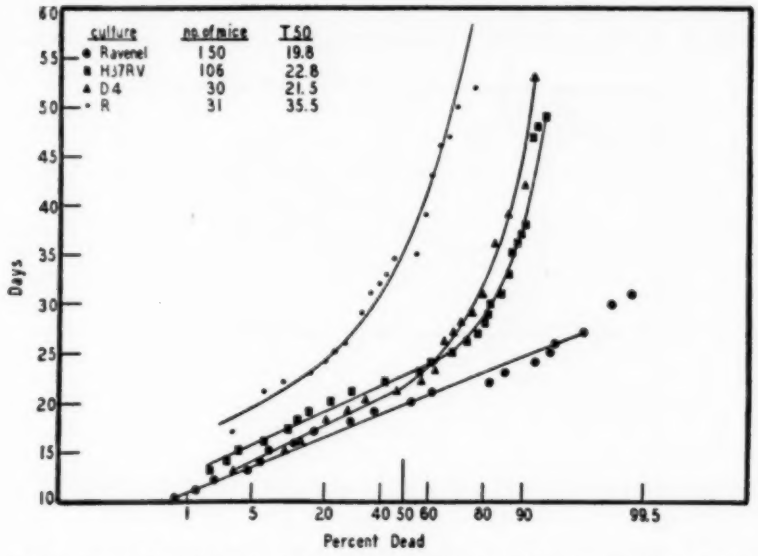


FIG. 1. Rates of death of mice infected with four strains of *M. tuberculosis* at a constant dose.

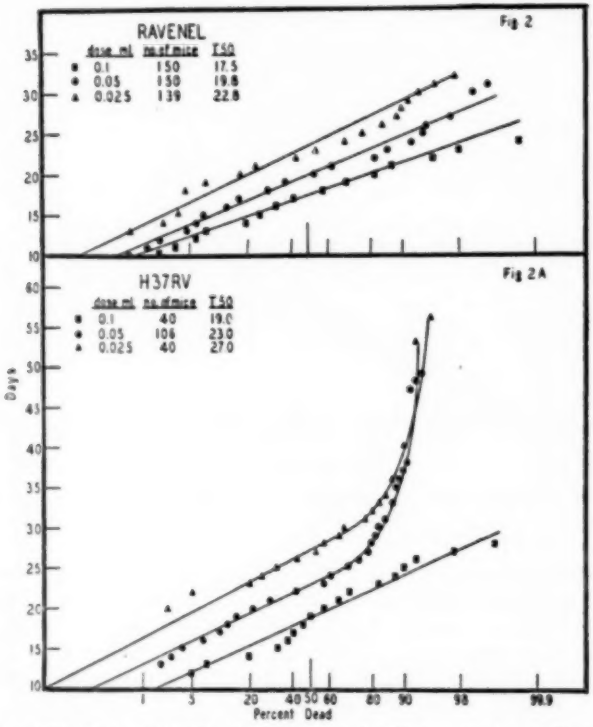
It is important in therapeutic tests that all control animals die regularly within a comparatively short space of time. The Ravenel culture best fulfills this requirement since it is rare for any mouse to survive beyond a 35 day period following infection with this strain. With the H37 Rv, D4, and R strains, 12, 20, and 45 per cent, respectively, of the mice survived beyond this period.

Since there might be some advantage in using a human strain and particularly one which has been used as widely as the H37 Rv, further experiments with this and the Ravenel strain were carried out. In these experiments two additional dose levels were used, that is, doses two and one-half times that previously used. All injections were given intravenously, the cultures being diluted so that the required dose was contained in 0.5 ml. The three doses, in terms of undiluted

culture, were 0.1, 0.05, and 0.025 ml. The numbers of mice infected with each dose of culture were as shown below.

DOSE OF CULTURE	NUMBER OF MICE	
	Ravenel	H37 Rv
ml.		
0.1	150	40
0.5	150	106
0.025	139	40

The per cent mortality on days 10 to 60 after infection is shown in figure 2. In this limited experience the 0.1 ml. dose of H37 Rv appears to be comparable



FIGS. 2 and 2A. Rates of death of mice infected with two strains of *M. tuberculosis* at three different dose levels.

to the 0.05 ml. dose of Ravenel and might be satisfactory for therapeutic experiments since all mice were dead before 30 days. Another factor, however,

must be considered. Since the 0.05 and 0.025 ml. doses of H37 Rv gave 12 and 15 per cent survivors, respectively, at 36 days there is little margin of safety if slight variation in virulence of culture should occur. With both the 0.05 and 0.025 ml. doses of Ravenel, the number of survivors was negligible.

The Ravenel strain was thus chosen tentatively as the standard culture for therapeutic studies. It was planned that this selection would be reviewed at a later date when actual therapeutic studies were run with drugs of known antituberculous activity in man (2).

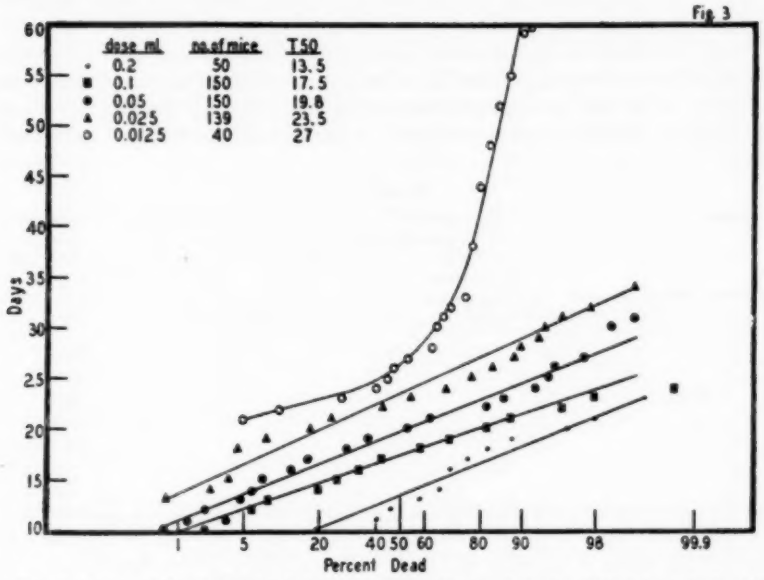


FIG. 3. Rates of death of mice infected with the Ravenel strain of *M. tuberculosis* at five different dose levels.

Standardization of Dose

The essential features of a standard dose are death of all control mice within a given time and uniformity of results in different experiments. Five different doses of the Ravenel culture have been studied. The results with doses of 0.1, 0.05, and 0.025 ml. have been presented in figure 2. These results with those of two additional doses, 0.2 and 0.0125 ml. are presented in figure 3. The numbers of mice in these two latter groups were 50 and 40, respectively.

Twenty-five per cent of the mice receiving 0.0125 ml. of the Ravenel culture survived beyond the 35th day, while with the other four doses either none or less than one per cent survived. The results obtained with 0.0125 ml. of the Ravenel culture are similar to those obtained with larger doses of cultures of lesser virulence. Since doses of 0.025 and 0.05 ml. both gave greater than 99

per cent mortality at 35 days, it was believed that the latter dose would give constant results even if slight variation in virulence of the culture should occur. A dose of 0.05 ml. (0.5 ml. of a 1:10 dilution) was consequently adopted as the standard for use in therapeutic experiments.

Saline vs. Egg Yolk as a Suspending Medium for Cultures

In the experiments which have been described, saline was used as the diluent for all cultures. Pierce, Dubos, and Middlebrook (27) have found that cultures of *M. tuberculosis* possess greater virulence for mice when 25 per cent egg yolk is used as a suspending medium.

Tests were made of the virulence for mice of *M. tuberculosis* Ravenel at five different dose levels when diluted in saline and in saline containing 25 per cent egg yolk. A 50 per cent egg yolk suspension was prepared and mixed with an equal volume of culture suitably diluted to give the final concentration desired.

TABLE 3

Enhanced Virulence for mice of M. tuberculosis Ravenel When Diluted in Saline Containing 25 per cent Egg Yolk

DOSE CULTURE	SALINE SUSPENSION		EGG YOLK SUSPENSION	
	Number mice	T_{50}	Number mice	T_{50}
0.2	10	14.6	10	10.8
0.1	10	15.8	10	12.5
0.05	32	18.5	30	16.2
0.025	10	22.4	10	18.0
0.0125	10	25.6	10	22.4

The diluted cultures were injected intravenously into mice in 0.5 ml. amounts. Ten mice were used for each dose except the 0.05 ml. dose in which case 32 mice received the saline suspension and 30 mice the egg yolk suspension.

The t_{50}'s for the 10 groups of mice may be seen in table 3.

With all doses of culture, the t_{50}'s for mice injected with the egg yolk suspensions were approximately 2½ to 4 days less than for those injected with saline suspensions. The time interval between first and last deaths was approximately the same for the groups injected with saline and egg yolk suspensions. Since the use of egg yolk rendered both the preparation of inoculum and the injection more difficult, however, it seemed better to adhere to the use of saline suspensions and achieve the desired survival time by control of dosage.

Maintenance of Virulence

Storage of culture at 3°C: As is frequently the case with cultures of other organisms, it was found that continuous serial transfer resulted in decrease in virulence, while cultures stored in the same medium at 3°C. for periods as long as eight to ten months retained virulence.

Weekly serial transfers of a culture were made during a four month period. During two months the virulence remained unchanged but during the third and and fourth months it gradually declined. At the end of four months the culture used to initiate the series, which had been stored at 3°C., was transferred to fresh medium and the virulence of the stored culture compared with that of the continuously transferred culture. Serial transfers from the stored culture were then continued for five months when its virulence also declined. These data are presented in table 4.

It is thus possible to maintain virulence in cultures stored in Kirchner-Tween 80 serum albumin medium at 3°C.

It was found that cultures stored at 3°C. for long periods of time grew slowly when transferred to fresh liquid medium. In order to determine what reduction in viable organisms occurred during long storage, serial dilutions of a culture stored for ten months were plated on Kirchner's agar containing 0.02 per cent Tween 80 and 0.25 per cent serum albumin. The counts indicated 430,000

TABLE 4

Comparison of Virulence of M. tuberculosis Ravenel When Continuously Transferred and When Stored at 3°C.

	CONTINUOUSLY TRANSFERRED JUNE 4 TO DECEMBER 31		STORED JUNE 4 TO OCTOBER 4 CONTINUOUSLY TRANSFERRED OCTOBER 4 TO FEBRUARY	
	Number mice	T_{50}	Number mice	T_{50}
June–July	130	19.3	Stored	
August–September	110	22.6	Stored	
October–December	100	25.4	120	19.4
January	Discontinued		60	19.5
February			30	26.3

viable organisms per ml. while fresh virulent cultures plated in this manner give an average count of 17 by 10^6 viable organisms per ml.

It was further found that large inocula of old cultures sometimes failed to initiate growth in fresh media but that growth followed the use of smaller inocula. This inability of a large inoculum to produce growth is illustrated in the following experiment: Ten-fold serial dilutions of three cultures, stored for one year, six months, and two weeks respectively, were made in a series of 5 tubes containing 5 ml. of Kirchner-Tween albumin medium. The tubes were incubated at 37°C. Turbidity readings were made at two to three day intervals. Growth did not occur in the 10^{-1} dilution of the culture stored for one year but did occur in dilutions 10^{-2} to 10^{-6}. The cultures stored for six months and two weeks grew in normal fashion, that is, tubes containing the larger inocula grew more quickly than those containing smaller inocula. The rapidity of growth of the three cultures was inversely proportional to the length of storage. The results are given in table 5.

A culture given a ten minute supersonic exposure showed the same inability

to grow in fresh medium when large inocula were used. It was further found that supersonic treatment of the Kirchner-Tween serum albumin medium rendered it incapable of supporting growth when inoculated with a culture of *M. tuberculosis*. It seems probable that slow hydrolysis of Tween 80 during a long period of storage at 3°C., or rapid hydrolysis due to supersonic treatment, releases enough free oleic acid to inhibit growth in fresh medium when large inocula are used.

Passage of culture through mice: Pierce, Dubos, and Middlebrook (27) maintain the virulence of cultures of *M. tuberculosis* by passage through mice. They readily obtained virulent cultures from lung, heart, or spleen tissue by cultivation in liquid medium.

TABLE 5

Growth of Three Cultures of M. tuberculosis Ravenel after Storage for One Year, Six Months, and Two Weeks

TURBIDITY	CULTURE-TIME OF STORAGE														
	One year					Six months					Two weeks				
	Culture dilution														
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
days															
2	Tr*	−	−	−	−	Tr	−	−	−	−	+	+−	−	−	−
4	Tr	−	−	−	−	2+	+−	−	−	−	2+	+	+−	−	−
7	Tr	−	−	−	−	4+	3+	+	−	−	4+	4+	2+	+	−
10	Tr	−	−	−	−	4+	4+	2+	−	−	4+	4+	3+	2+	+−
13	Tr	+	+−	−	−	4+	4+	3+	+	+−	4+	4+	3+	3+	2+
16	Tr	2+	2+	+	−	4+	4+	3+	2+	2+	4+	4+	4+	3+	3+
19	Tr	4+	4+	3+	+	4+	4+	4+	4+	3+	4+	4+	4+	4+	4+

* Tr (trace) in the 10^{-1} dilution of cultures indicates turbidity due to amount of original culture present and not to growth.

Blood and tissue suspensions of mice infected with *M. tuberculosis* Ravenel were cultured on Loewenstein's medium, and were inoculated into Kirchner's medium, and Kirchner's containing Tween 80 and serum albumin. Difficulty was found in obtaining positive cultures in the medium containing Tween 80. This was found to result from the fact that mouse serum and tissue contain a lipase which hydrolyzes Tween 80 with the production of free acid inhibitory to the growth of the tubercle bacillus. In a further study of the sera of normal animals, it was found that guinea pig and mouse sera were equally potent in hydrolyzing Tween 80. Rabbit serum also contained lipase but to a lesser degree. Davis and Dubos (25) have shown that horse serum also contains a lipase capable of hydrolyzing Tween 80. Positive cultures were obtained, however, in Kirchner's medium and on Loewenstein. Transfers from these media were made to Kirchner-Tween 80 serum albumin medium for virulence test. Eight cultures obtained from the blood or organs of 3 different mice have been so tested. In table 6 is shown the high virulence of all of these cultures.

When serial passage in mice was tried, it appeared that the virulence increased through four passages. The fifth passage, however, proved to have about the same virulence as the first. Passages were not made directly from mouse to mouse by use of tissue suspensions but by infecting each mouse in series with cultures grown on artificial medium obtained from the previous mouse. Browning and Gulbransen (11) tested the effect on virulence of direct passage from mouse to mouse. They used 8 strains with 3 to 8 passages each and concluded that, while virulence repeatedly appeared to increase, there was no maintenance of such increase.

It thus appeared that results obtained by mouse passage were not sufficiently uniform to render advisable the use of this method for maintenance of virulence.

Employment of cultures derived from rough colonies: Dubos, Davis, Middlebrook, and Pierce (23) and Middlebrook, Dubos, and Pierce (28) have described

TABLE 6

Virulence of Culture of M. tuberculosis Ravenel Recovered from Infected Mice

CULTURE RECOVERED FROM		DOSE	NUMBER MICE	T_{50}
Mouse	Tissue			
		ml.		
1	Blood	0.05	20	18.8
2	Heart	0.05	10	18.8
	Lung	0.05	20	17.4
	Spleen	0.05	10	15.4
3	Blood	0.05	20	17.6
	Heart	0.05	10	14.8
	Spleen	0.05	10	19.0
	Lung	0.05	20	16.4

the growth of virulent and avirulent cultures of *M. tuberculosis* on the surface of a Tween albumin agar medium. In order to get satisfactory growth, they found it necessary to use a 0.5 per cent concentration of serum albumin. On this medium colonies developed within seven to fifteen days. They found that the type of colony of both virulent and avirulent bacilli changed from rough to smooth as the concentration of Tween 80 in the medium was increased. Virulent bacilli, however, retained their rough characteristics in higher concentrations of Tween than did the avirulent bacilli.

This method of colony differentiation into virulent and avirulent types appeared to offer an excellent opportunity to obtain highly virulent cultures derived from single rough colonies. Using Kirchner's medium as a base with the addition of 1.5 per cent washed agar, Tween 80 was added in 0.005, 0.01, 0.02, and 0.05 per cent. To this agar containing each concentration of Tween 80, bovine serum albumin, fraction V, was added in 0.1, 0.2, and 0.5 per cent and plates were poured. The plates were inoculated with 0.1 ml. of 10^{-4} and 10^{-5}

dilutions of *M. tuberculosis* Ravenel grown in Kirchner-Tween 80 serum albumin medium. The inoculum was allowed to spread by gently tilting the plates which were then sealed with cellulose tape and incubated at 37°C.

In confirmation of Dubos' results, predominantly rough colonies appeared on the plates containing 0.005 and 0.01 per cent Tween 80 and an increasing proportion of smooth colonies on the agar containing 0.02 and 0.05 per cent Tween

TABLE 7

Comparative Virulence of Cultures Derived from Smooth and Rough Colonies of M. tuberculosis Ravenel

CULTURE	COLONY TYPE	DOSE	COLONY COUNT MILLION/ML.	NUMBER MICE	T50	
		ml.				
1	Rough	0.05	not made	10	17.3	
	Smooth	0.05	not made	10	37.0	
2	Rough	0.05	16.5	20	18.6	
	Smooth	0.05	70.0	18		11 died 23 to 76 days 7 killed 86th day
3	Rough	0.05	9.0	20	20.1	
	Smooth	0.05	39.7	10		3 died 50 to 160 days 7 killed 160th day
4	Rough	0.05	not made	10	20.0	
	Smooth	0.05	not made	10		5 died 35 to 81 day 5 killed 129th day
5	Rough 1	0.05	6.0	10	20.2	
	Rough 2	0.06	not made	10	17.8	
	Smooth 1	0.05	93.0	10		6 died 17 to 108 days 4 killed 122nd day
	Smooth 2	0.05	not made	10		10 killed 121st day
6	Rough 1	0.05	7.0	10	20.0	
	Rough 2	0.05	7.5	10	20.0	
	Smooth 1	0.05	54.5	10		8 died 26 to 72 days 2 killed 122nd day
	Smooth 2	0.05	75.0	10		3 died 69 to 108 days 7 killed 122nd day

80. The concentration of serum albumin appeared to have little influence on colony type. With 0.05 per cent Tween 80, however, growth was slightly inhibited in the presence of 0.1 per cent but not in the presence of 0.2 and 0.5 per cent serum albumin.

In later work, Kirchner agar containing 0.02 per cent Tween 80 and 0.25 per cent bovine serum albumin was used. On this agar colonies appear as rough, intermediate, or smooth. The rough colonies are flat and spreading, have a small dense center and a delicate lacy border. The smooth colonies are denser

and more uniform in appearance, with a glistening surface and a well defined edge.

Both rough and smooth colonies have been picked from 6 different Ravenel cultures, plated on Kirchner's agar containing 0.02 per cent Tween 80 and 0.25 per cent bovine serum albumin. These colonies were transferred to Kirchner-Tween 80 serum albumin fluid medium and, when heavy growth occurred (usually in about seven days), transfers were made to fresh medium in the usual manner. After incubation for five days, the cultures were tested for virulence. In table 7 the high virulence for mice of the rough colony cultures in contrast to the very low virulence of the smooth colony cultures is shown.

From these experiments, it is clear that virulence is associated with the rough colony type which develops when the cultures are grown on agar containing 0.02 per cent Tween 80 and 0.25 per cent serum albumin.

In a further study, 21 cultures derived from rough colonies have been tested for virulence. Seventeen out of the 21 cultures gave $t_{50\text{-}\%}$ varying between 17.6 and 21.6 days with an average of 19.7 days, while the $t_{50\text{-}\%}$ of the 4 cultures of lesser virulence varied between 23 and 25 days. Twenty-six virulent cultures, transfers from the original culture and maintained in Kirchner-Tween 80 serum albumin medium at 3°C., gave $t_{50\text{-}\%}$ varying between 16.8 and 21.6 days with an average of 19.7 days.

The above cultures, at the time virulence tests were made, were all plated in order to study the correlation between virulence and proportion of rough and smooth colonies. The results have been disappointing. Colony differentiation was not always clear-cut, and unexplained differences occurred in colony form on duplicate plates made from the same culture. Studies are now in progress to develop a technique by which more consistent results can be obtained.

Standard Procedure for Maintaining Cultures of M. tuberculosis Ravenel for Mouse Infection

For the purpose of obtaining rough colony cultures to be used for mouse infection, a virulent culture of *M. tuberculosis* Ravenel is plated on Kirchner's agar containing 0.02 per cent Tween 80 and 0.25 per cent bovine serum albumin, fraction V. (A 5 per cent serum albumin solution made in distilled water and sterilized by filtration is added to the melted agar which has been cooled to 56°C.) Four to six plates containing 15 ml. of the above agar are inoculated with 0.1 ml. of a 10^{-4}: dilution of culture. The inoculum is placed on the surface of the agar and allowed to spread by gently tilting the plates which are then sealed with cellulose tape and incubated at 37°C. for twenty-one days. Fresh cultures are started from rough colonies picked from these plates and such cultures, after two transfers in Kirchner-Tween serum albumin medium, are injected into mice and stored at 3°C. If found to be of satisfactory virulence they are available for use as seed cultures.

The cultures to be used for mouse infection in the actual tests are inoculated with the seed cultures obtained as described above. These cultures, which are grown in 50 ml. Erlenmeyer flasks containing 20 ml. of Kirchner's medium with

0.05 per cent Tween 80 and 0.1 per cent human serum albumin, are started by an inoculum of 2 ml. of the virulent, stored culture, and incubated for five days at 37°C. As a check on purity of the culture, a transfer is made after two days' incubation to beef heart broth. In this medium the tubercle bacillus does not grow but the usual contaminants grow well. As a further check on the purity of the culture, an aliquot is examined by the Ziehl-Neelsen method just before use. Turbidity of the culture is then determined by comparison with McFarland (29) nephelometer tubes. With a standard procedure for maintaining cultures, the turbidity of the virulent Ravenel culture is fairly constant. It has been observed, however, that, when cultures lose virulence, growth becomes heavier. The organisms of lesser virulence evidently reproduce more rapidly under the standard conditions described. If found to be of standard turbidity, the culture is diluted 1:10 with saline and injected intravenously in 0.5 ml. amounts.

SUMMARY

A procedure for maintaining cultures of *M. tuberculosis* of standard virulence for use in therapeutic experiments in mice has been established.

The standard medium chosen for growth of cultures was Kirchner synthetic medium with the addition of 0.05 per cent Tween 80 and 0.1 per cent human serum albumin.

A bovine strain of *M. tuberculosis*, the well-known Ravenel, was chosen as the standard strain since it proved more virulent and gave more constant results than three other strains tested.

The Ravenel culture when maintained in this medium under the conditions described retains its virulence for mice and gives uniform results in successive experiments. A five day culture when diluted 1:10 with saline and injected intravenously in 0.5 ml. amount gives greater than 99 per cent deaths within 35 days with an average t_{50} of 19.8 days.

SUMARIO

Empleo del Ratón en una Prueba Normalizada de la Actividad Antituberculosa de los Compuestos de Origen Sintético Natural. I. Selección y Normalización del Cultivo

Queda establecido un procedimiento para mantener cultivos del *M. tuberculosis* de virulencia tipo para empleo en los experimentos terapéuticos en ratones.

El medio tipo escogido para los cultivos fué el sintético de Kirchner con la adición de 0.05 por ciento de "Tween 80" y 0.1 por ciento de seroalbúmina humana.

Como cepa tipo se tomó una cepa bovina del *M. tuberculosis*, la conocida Ravenel, por resultar más virulenta y dar resultados más constantes que otras tres cepas comprobadas.

El cultivo Ravenel, mantenido en dicho medio en las condiciones descritas, retiene su virulencia para los ratones y rinde resultados uniformes en experi-

mentos sucesivos. Un cultivo de cinco días, diluído al 1:10 con solución salina e inyectado intravenosamente en cantidades de 0.5 ml., provoca más de 99 por ciento de muertes en término de 35 días con un promedio de t_{50} de 19.8 días.

Acknowledgment

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THE USE OF THE MOUSE IN A STANDARDIZED TEST FOR ANTITUBERCULOUS ACTIVITY OF COMPOUNDS OF NATURAL OR SYNTHETIC ORIGIN[1]

II. Choice of Mouse Strain

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INTRODUCTION

Despite its widespread use in the study of chemotherapy of many experimental diseases, the mouse has been extensively used for therapeutic evaluations in experimental tuberculosis only in relatively recent years. Hesitancy over use of this animal arose for several reasons, the chief one of which was the impression that this species was highly resistant to infection. Undoubtedly this belief grew up from work done early in this century in which the intraperitoneal or subcutaneous routes of inoculation were employed. Much of this early work has recently been reviewed by Raleigh and Youmans (1a) and detailed reference to it need not be made here, except perhaps to put additional emphasis on differences in various mouse strains in their susceptibility to experimental infection with this agent.

Koch (2) reported the very early observation that field mice (*Arvicola arvalis*) were more susceptible than house mice. Although studies employing various strains of the house mouse (*Mus musculus*) have been described during the ensuing years, little has been said about strain differences in susceptibility. This problem was attacked recently by Pierce, Dubos, and Middlebrook (3). Their work was facilitated by the improved cultures obtained by the use of a dispersing agent (Tween) in their culture medium. Of 22 strains of mice of varying degrees of homozygosity from various sources which they tested, they listed 18 in the following order of increasing susceptibility to tuberculous infection: Swiss albino (4 strains from different sources), Swiss albino *CFW*, Swiss albino CFCW, Strong A, Strong C, Buffalo, Rockefeller Institute strain, Chocolate, Brown spot, C_3H (strains from two sources), Strong I, *Mus musculus* domesticus (raised in Rockefeller Institute), C_{57} black, and dba (line 1). Thus in general, pigmented animals were found to be more susceptible than albino strains.

During the past few years similar studies have been carried out in this laboratory. One point of especial emphasis has been to seek a strain of mouse with not only high susceptibility but also with as uniform a response as it was possible to find. Such characteristics would help greatly to evaluate various compounds

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of possible therapeutic value. It was recognized at the outset that the most susceptible strains might not necessarily be the most uniform in response.

Martin (4) presented an analysis of his data on the rate of death of mice following a fixed intravenous inoculation, but it is not clear as to what type or strain of mouse he used. Deaths occurred between the fifteenth and thirty-first days following inoculation, with a peak of deaths occurring on the nineteenth and twentieth days, and with approximately 5 per cent surviving through the thirty-first day. In two experiments described by Youmans and McCarter (5) 40 per cent of the control mice survived through the twenty-eighth day in one case, while only 13 per cent survived this period in the second. In both experiments the earliest deaths occurred on the seventeenth or eighteenth day. These authors described their mice as being white Swiss of about 25 Gm. weight. In later work, Youmans and Raleigh (1b, c) employed the Strong A strain of albino mice averaging 6 to 8 weeks in age, usually weighing between 20 and 25 Gm. The inoculum consisted of 0.1 mg. of *M. tuberculosis*. The rate at which the mice died following inoculation was not reported but apparently there was considerable variation, since survival times ranging from 14 to as long as 94 days were mentioned with an average of about 25 days (1b). Probable reasons given for the differences were variations in the inoculating culture as well as in individual resistance of mice. On the latter score it may be noted that Pierce, Dubos, and Middlebrook (3) listed the Strong A strain of mice as amongst the more resistant tested.

Raleigh and Youmans (1b) mentioned also the possibility that development of bacterial hypersensitivity may play a role in variations. According to Martin (4), mice receiving a small inoculum may show marked increases in survival time "due to the rapid development of a measure of resistance."

EXPERIMENTAL

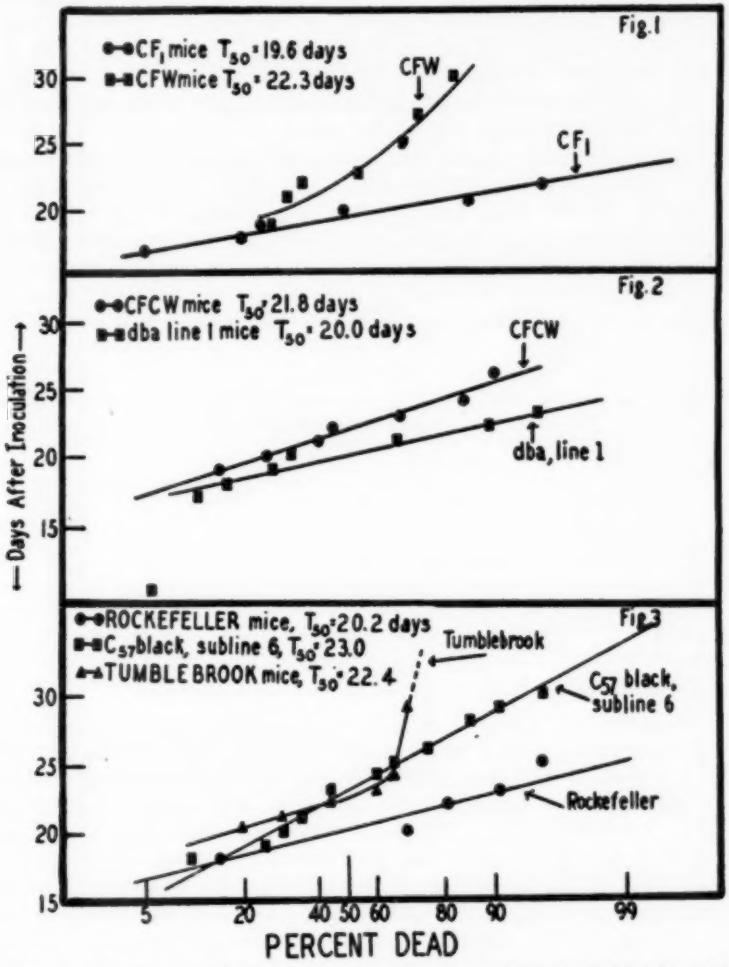
The prime purpose of the work to be described here was to find a strain of mice giving the most uniform response possible. Relatively short survival time was the second consideration. The standardization of inoculum for these tests was described in a previous paper (6) and for the reasons there given the Ravenel strain of *M. tuberculosis* var. bovis has been employed throughout.

Preliminary studies were carried out with 7 strains of mice, as follows: CF1 (Carworth Farms albino with brown, non-agouti background), CFW (Carworth Farms albino, agouti), Rockefeller (Princeton) albino, Tumblebrook (Swiss Webster), CFCW (CFW mutant), dba (line 1), and C_{57} black, sub-line 6 (the latter two are inbred strains from the Jackson Memorial Laboratory, Bar Harbor, Maine. The C_{57} strain is black, non-agouti, and the dba is a dilute brown, non-agouti.)

The Ravenel strain of *M. tuberculosis* grown in a modified Kirchner's medium containing Tween 80 and serum albumin, under standard conditions as described previously (6), was diluted to a standard turbidity and the mice under study were given an intravenous inoculation of 0.5 ml. of the diluted culture. (Usually this consisted of a 1:10 dilution of a five day culture, containing on an average

8 by 10^5 viable organisms, as indicated by plate counts, or the equivalent of approximately 0.0065 mg. of organisms (washed and dried weight).

The death rates of the various strains of mice following inoculation with *M. tuberculosis* (Ravenel) are shown in figures 1, 2, and 3 in which the cumulative percentage dead is plotted on a probability scale against time on an arithmetic scale.[2] It will be noted that with 2 Swiss strains (CFW and Tumblebrook),



FIGS. 1, 2, 3. Rates of death of mice experimentally infected with *M. tuberculosis*.

[2] The reasons for using this type of graph are discussed later in this paper.

such graphs deviated significantly from a straight line, with early deaths occurring at rates similar to those of other strains tested, while some mice survived longer than might be expected according to a normal frequency distribution. It is believed that this indicates a lack of homogeneity in response on the part of these strains to experimental infection with *M. tuberculosis*. The remaining 5 strains all died at rates giving straight lines on probability paper indicating that death times followed a normal frequency distribution curve. From these plots it is possible to determine the 50 per cent death times (t_{50}). A comparison of the 7 strains of mice on this basis is shown in table 1.

Since these preliminary studies were carried out with groups of only 20 mice each, no particular significance can be put on the exact order in which the t_{50} values appear. It was somewhat surprising, nevertheless, to find that the C_{57} blacks apparently were somewhat more resistant than some of the albino strains. It will be recalled that Pierce, Dubos, and Middlebrook (3) had found this strain (sub-line not indicated) to be one of the most susceptible to the H37 Rv strain of *M. tuberculosis*. Unfortunately, the Bar Harbor disaster prevented the further investigation of this point. In recent months it has again become possible to obtain mice of the C_{57} black strain, sub-line 6, and 40 additional mice (20 mice in each of two tests) were inoculated under standard conditions along with CF1 mice as controls. The results of the more recent duplicate tests agreed with each other very well, but did not agree completely with previous findings. Thus while the CF1 mice gave a composite t_{50} of 19.7 days (average survival time = 20.2 days), the C_{57} blacks gave a t_{50} of 20.7 days (average survival time = 21.7 days) indicating on this score a greater similarity in sensitivity between the two strains than had been found in earlier tests. However, again the CF1 mice showed a greater uniformity in response in that all deaths occurred between the sixteenth and twenty-fifth days following inoculation while in the C_{57} blacks, deaths spread out from the thirteenth day through the thirty-ninth day.

TABLE 1

Comparison of 50 per cent Death Times (t_{50}) of Various Strains of Mice Experimentally Infected with M. tuberculosis (Ravenel)

MOUSE STRAIN	T_{50}
	days
CF1	19.6
dba, line 1	20.0
Rockefeller	20.2
CFCW	21.8
CFW	22.3
Tumblebrook	22.4
C_{57} black, sub-line 6	23.0

In general it appeared that the various strains of mice tested fell into two groups. One consisted of animals showing remarkable homogeneity of response to this experimental infection. In this group could be placed the CF1, CFCW,

dba, and C_{57} black strains. In the second group, in which the CFW and Tumblebrook strains could be placed, the individual members showed greater variation in response to this infection. It will be noted that such a rough classification would put the CFW mouse in one group, and a mutant of this strain, the CFCW in another, but to speculate further at this point would be extremely hazardous. Time-mortality curves of a relatively long term infection such as that with *M. tuberculosis* are undoubtedly the results of the composite effects of many complex factors. It was felt essential, however, that more extensive studies be carried out on much larger groups of mice. For this purpose two strains were chosen, one strain from each of the two groups described. The CF1 strain, because of ready availability, was picked to exemplify the homogeneous group, while the CFW strain was chosen as an example of the heterogeneous group. Eleven separate tests, employing a total of 149 mice, were

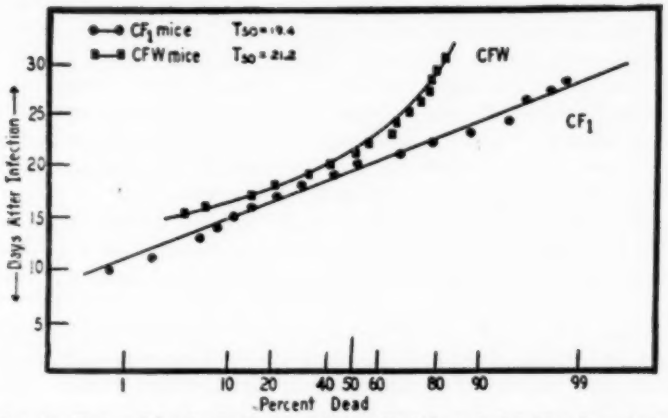


FIG. 4. Rates of death of mice experimentally infected with *M. tuberculosis*.

carried out with CF1 mice; and 14 tests, totaling 139 mice, with the CFW. As before, all mice received a similar, standardized, intravenous inoculation with *M. tuberculosis* (Ravenel).

For the sake of brevity, the composite results of all of these tests are shown graphically in figure 4 which shows the time-mortality curves for the two strains. As had been found in the preliminary studies, experiments with large groups of mice showed that the CF1 strain was significantly more uniform in response than the CFW to the standard infection with *M. tuberculosis* (Ravenel). Accordingly the CF1 strain was chosen as the standard mouse to be used in chemotherapeutic studies, a description of which appears in the third paper of this series (7).

The Choice of Route of Inoculation

Thus far the discussion has been limited to the response of various strains of mice following infection via the intravenous route. Other routes of infection in

mice have been employed in various laboratories. The Royal Commission on Tuberculosis (8) found in its very early work that rats and mice were relatively resistant, especially to small doses, when *M. tuberculosis* was administered intraperitoneally, subcutaneously, or fed in the diet. A number of workers have employed the respiratory route of infection. Glover (9), for example, reported that a dose of about 100 organisms would infect some mice, 1,000 organisms infected nearly all, but the minimal lethal dose was estimated at about 120,000 organisms. Intranasal inoculation gave less consistent results than exposure to an infected mist. Concentrated aerosol mists induced intensive pulmonary consolidation within 60 days. Weaker suspensions were not lethal, but caused discrete, slowly progressive foci. The strain, age, and weight of mice used were not indicated.

Pierce, Dubos, and Middlebrook (3) have studied tuberculosis in mice following inoculation via the intravenous, intraperitoneal, and intracerebral routes. The minimal infective dose was said to be 0.0005 to 0.02 mg. of bacilli by the first route. The second avenue of infection (intraperitoneal) was much less effective, although it could be improved by using as inoculum a culture suspended in egg yolk. In the intracranial route, as few as 10 to 100 bacilli, it was estimated, gave rise to very rapid growth of the organism. It is not possible, however, to make a comparison between the death rates following intravenous and intraperitoneal inoculation since these data are not given for the latter route.

The several routes of inoculation were taken into consideration in the present work. The intranasal inoculation presents undue hazards for the laboratory worker, especially when employed in air-conditioned buildings, and therefore was eliminated on this score.

On the basis of the report of Pierce, *et al.* (3), it would appear that intracerebral inoculation may prove to be a convenient method for detection of *M. tuberculosis* in sputum since very small inocula are detectable by this means. Although pulmonary lesions were noted 3 weeks after infection, it was not clear from the data, as already noted above, at what rate deaths followed inoculation by this route. Since the present studies were designed primarily for chemotherapeutic tests, many of which would involve the use of new, and possibly poorly defined, substances, consideration had also to be given to the question of whether or not any substance under test would pass through the blood-brain barrier following administration parenterally or by mouth. As this type of information would not always be available, it was felt that the intracranial route might lead to undue complications for this purpose. Hence comparisons were made of intraperitoneal (employing yolk fluid as a suspending medium) and intravenous inoculation. The results of typical experiments of this type are shown in table 2.

It is apparent that whether or not egg yolk is used as the diluent, intravenous inoculation leads to considerably more rapid death in mice than does intraperitoneal inoculation even when the inoculum used via the latter route is ten times as large as that used in the former.

It is also evident that the use of egg yolk accelerates the death rate following intravenous inoculation, as compared to inocula prepared in saline. However,

on the basis of results with admittedly small numbers of mice, it appeared that the use of egg yolk for intravenous inoculation did not lead to any more reproducible results than did using saline-suspended inocula. Hence it was decided not to use egg yolk in the routine chemotherapeutic tests to be described in a third paper of the present series (7).

The Effect of Age, Weight, and Sex of Mice Used in Experimental Tuberculosis Experiments in CF1 Mice

After the test had been standardized as to strain of *M. tuberculosis* (6), diluting fluid of inoculum, strain of mice, and route of inoculation, attention was turned to the question of the effects of age, weight, and sex of the CF1 mice on the death rate following a fixed inoculation. It is evident that age and weight are some-

TABLE 2

Comparison of Intravenous and Intraperitoneal Inoculation of CF1 Mice with M. tuberculosis (Ravenel)

NUMBER OF MICE	INOCULUM		ROUTE	T_{50}	AVERAGE SURVIVAL TIME
	Diluent*	Dose† Ml.			
42	25 per cent yolk	0.05	IV	16.1	15.3
30	0.9 per cent saline	0.05	IV	19.6	19.9
29	25 per cent yolk	0.5	IP	22.5	24.4
10	25 per cent yolk	0.25	IP	27.5	35.7
10	25 per cent yolk	0.125	IP	35.8	39.1

* Final concentration of diluting material.

† Dose given in terms of undiluted 5 day culture. For intravenous inoculation the dose was diluted to 0.5 ml. with the given diluent while for intraperitoneal injection the final volume was 1.0 ml.

what interdependent and there are limitations as to the age limits obtainable within a given weight range. However, as shown in table 3, which summarizes the results of a number of experiments, it can be seen that CF1 mice of either sex, weighing between 14 and 18 Gm. and ranging in age from 4 to 6 weeks all respond in a very similar fashion to the standard intravenous inoculation with *M. tuberculosis* (Ravenel) as used in the present tests. On the other hand, amongst CF1 mice 10 weeks of age or older, deaths following the standard inoculation occur not only more slowly, but also the rates deviate considerably from a normal frequency distribution curve and hence do not give straight lines on probit paper as described above.

The Choice of Suitable End Points in Experimental Tuberculosis in the Mouse

One difficulty which has always been present in studies in experimental tuberculosis in animals has been that of interpretation of results once they have been obtained. Feldman (10) has described a numerical scheme for recording the degree of tuberculous changes occurring in the experimentally infected guinea pigs. In a later paper (11) it was emphasized that one of the chief problems in

the search for chemotherapeutic compounds for tuberculosis was the difficulty in distinguishing definitely between effective and noneffective substances in a test of only short duration.

In using the mouse for assessment of therapeutic action of various compounds in tuberculosis, Martin (4) used the increase in the arithmetic mean survival time of treated mice as compared to infected, but untreated, controls. He noted that when more than 5 out of 24 mice survived beyond the end of the experiment (30 days) a better measure of therapeutic efficacy could be obtained by comparison of median survival times, *i.e.*, the times taken for 50 per cent of the animals in the various groups to die. The median survival time was estimated from a smoothed probit-cumulative distribution curve. Although the difference in reliability between the two methods was found to be small, the use of the mean survival time was considered more reliable.

Youmans and Raleigh (1c) employed several criteria for measuring therapeutic

TABLE 3

Effect of Age, Weight and Sex on Rate of Death of CF1 Mice Following Intravenous Inoculation with M. tuberculosis (Ravenel)

SEX	AGE WEEKS	AVERAGE INITIAL WEIGHT	NUMBER OF MICE	T_{50}	AVERAGE SURVIVAL TIME
		Gm.		*Days*	*Days*
♂	4 to 5	16.2	152	20.3	21.0
♀	4 to 5	15.2	153	20.1	20.5
♂	5 to 6	17.7	153	20.0	20.7
♀	5 to 6	16.4	154	19.3	20.3
♂	10 to 12	24.0	29	25.1	28.3
♀	10 to 12	23.2	20	25.0	27.6
♀	6 months	24.8	8	36.0	38.0

action of various compounds in experimental tuberculosis in the mouse. These criteria included average survival time, per cent mortality, weight response, and gross and microscopic tuberculous involvement of the tissues. Apparently, no attempt was made to compose these separate operations into comparative indices or ratios of activity and it is not clear how two drugs would be compared on a relative basis. In discussing Martin's procedure of comparing drug activities solely on the basis of average survival times, these authors expressed the feeling that in experimental tuberculosis in the mouse direct examination of tissues for suppression of lesion development was a "necessary measure of chemotherapeutic action."

In the earlier stages of the work on experimental tuberculosis in the mouse in our laboratory, attempts were made to employ, as one of the criteria, the degree of tuberculous involvement of the various tissues. This will be discussed more fully in the third paper of the present series (7). The present paper will limit itself to a discussion of the death rates of the CF1 mice following the standardized inoculum.

Examination of death rates of CF1 mice infected with standardized *M. tuberculosis* (Ravenel) inocula in many replicate tests carried out over several years time indicate that, when these mice receive no therapeutic treatment, the cumulative percentage dead plotted on a probability scale against time in days on an arithmetic scale[3] gives a straight line. This indicates that the death-time relationship follows a normal frequency distribution curve. Using what are apparently somewhat less susceptible mice, Raleigh and Youmans (1b) also observed this type of distribution. It is implied also in the data published by Martin (4). The extent to which such curves deviate from a straight line on probit paper is a measure of the variation from this normal distribution and, as indicated earlier, has been taken to be a measure also of the uniformity of response of a given group or strain of mice. It will be recalled that Bliss (12) calculated time-mortality curves in various biological systems and employed in his graphic analysis plots of survival time on a logarithmic scale against mortality in probits. Furthermore, Bliss computed curves statistically from the same data and showed that using log time the provisional curve (obtained by plotting experimentally observed mortality in probits against log time) agreed closely with the computed curves. Such computations have not been carried out with time-mortality data obtained from studies of experimental tuberculosis in the mouse. As already noted, however, it was found that straight lines are obtained by plotting cumulative per cent dead on a probability scale (which is the equivalent of using probits) against time on an arithmetic scale. Using log time does not appreciably improve the nature of this line nor does it significantly change the estimated t_{50}. Thus, when the data obtained with CFW mice (figure 4) are plotted, using log time instead of arithmetic time, there is a deviation from a straight line above 65 per cent mortality, although this deviation is less marked since the log time scale condenses the points with increased time.

It should be pointed out, however, that Bliss, in addition, provided a procedure which, though laborious, did permit calculation of the error in estimating the time in which any per cent mortality desired had occurred. Any advantages which may be derived from making comparisons on a basis other than the 50 per cent mortality time are not yet obvious in our present work. Hence the t_{50} (50 per cent mortality time) has been used as the chief "end point" criter on in these studies.

Early in the work it was decided that it would be of value to compare t_{50} values (obtained by plotting on arithmetic-probability paper as already described) with average survival times. In table 4 such data from 12 separate experiments are listed. In each case 10 to 20 CF1 mice receiving the standard intravenous inoculum were employed.

It is obvious that, under the experimental conditions employed, the values for the estimated t_{50}'s and the average survival times coincided very closely. The standard deviation from test to test for the t_{50} value was 1.97 days, or 10.3

[3] A convenient type of graph paper for this purpose is the arithmetic-probability chart sheet such as that printed by the Codex Book Company of Norwood, Massachusetts, under their catalogue number 3127.

per cent, while for the average survival time it was 1.85 days, or 9.26 per cent. The advantage in using the t_{50} from a practical standpoint lay in the ability to estimate the t_{50} before 100 per cent of the mice had died. Also, in those occasions when deaths occurred erratically, it was obvious from the nature of the curve obtained that the test was atypical.

Furthermore, it was anticipated that treatment with therapeutic agents would prolong the lives of some individual mice to a much greater extent than others. One or two mice out of 10 living much longer than the rest could lead to a significantly changed value for the average survival time without appreciably changing the t_{50}. Hence, as a standard practice t_{50} values were employed in preference to average survival times.

TABLE 4

Comparison of t_{50} and Average Survival Time in CF1 Mice Inoculated with M. tuberculosis (Ravenel)

EXPERIMENT NUMBER	T_{50}	AVERAGE SURVIVAL TIME
	Days	*Days*
1	18.2	18.8
2	19.8	19.8
3	18.3	18.7
4	17.7	20.4
5	20.8	21.4
6	17.5	18.5
7	13.7	15.7
8	18.4	18.8
9	20.3	21.0
10	20.8	22.1
11	21.0	22.1
12	21.0	22.3

SUMMARY

Using a carefully standardized inoculum of *M. tuberculosis* var. bovis (Ravenel) grown in a liquid medium containing Tween 80, comparisons have been made of the death rates of 7 strains of mice. The mouse of choice on the basis of uniformity of response, as well as ready availability in large supply, was found to be an albino mouse with brown, non-agouti background, the CF1. Intravenous inoculation was shown to be preferable to the intraperitoneal route even when egg yolk was used as the suspending medium for the inoculum. As was the case with the selection of the Ravenel strain of *M. tuberculosis* var. bovis, the choice of the mouse was regarded as tentative. Final choice could only be made when results were available on tests in this animal of drugs with known antituberculous activity in man (7). The most uniform results were obtained with CF1 mice weighing between 14 to 18 Gm. and ranging in age from 4 to 6 weeks.

Deaths in CF1 mice of appropriate age receiving a standard intravenous inoculum followed a normal frequency distribution curve which gave straight lines when cumulative percentage mortality, on a probit scale, was plotted against

time on an arithmetic scale. Such straight lines provide a relatively simple procedure for determining the 50 per cent mortality time (t_{50}). Among infected CF1 mice receiving no therapeutic treatment, there was little difference between t_{50} and average survival times. For certain practical considerations, however, the t_{50} was considered to be preferable to the average survival time as an end point criterion in a test designed for chemotherapeutic studies.

SUMARIO

Empleo del Ratón en una Prueba Normalizada de la Actividad Antituberculosa de los Compuestos de Origen Sintético Natural. II. Selección de la Cepa Murina

Tomando un inóculo cuidadosamente normalizado de *M. tuberculosis*, var. *bovis* (Ravenel), cultivado en un medio líquido que contenía "Tween 80," se compararon los coeficientes de mortalidad de siete cepas de ratones. A base de uniformidad en los resultados, así como de accesibilidad en grandes cantidades, el ratón de elección resultó ser un ratón albino, con un fondo pardo, no-agutí, el CF 1. La inoculación endovenosa se mostró preferible a la vía intraperitoneal, aun al usar yema de huevo como medio suspensor para el inóculo. Lo mismo que con la selección de la cepa Ravenel del *M. tuberculosis* var. *bovis*, la elección del ratón fué considerada como tentativa, pues sólo cabe hacer la selección definitiva cuando se cuente con el resultado de las pruebas verificadas en este animal con drogas de conocida actividad antituberculosa en el hombre (7).

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THE USE OF THE MOUSE IN A STANDARDIZED TEST FOR ANTITUBERCULOUS ACTIVITY OF COMPOUNDS OF NATURAL OR SYNTHETIC ORIGIN[1]

III. The Standardized Test

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INTRODUCTION

In previous papers (1, 2) information has been presented on data leading to the selection of the Ravenel strain of *M. tuberculosis* var. bovis and the CF1 homozygous strain of albino mice for use in a standardized test for antituberculous activity of compounds. These reports contained, in addition, evidence as to the best means of maintaining the culture as well as to the optimal dose of culture and route of administration.

The above information has been utilized in the establishment of a standardized *in vivo* test by which compounds of both natural and synthetic origin are tested both orally and parenterally for activity against infection due to *M. tuberculosis*. Such *in vivo* tests for activity are carried out on compounds first selected on the basis of tests *in vitro* and at doses indicated by preliminary tests for toxicity, or rather, acceptability, by the mouse.

The standard techniques, together with some characteristic results will be described below.

MATERIALS AND METHODS

The strain used is the Ravenel strain of *M. tuberculosis* var. bovis. The reasons for the choice of this strain, together with the standard methods of transferring and maintaining the culture so as to assure constant and maximal virulence and of preparing the infecting inoculum, are given elsewhere (1). Similarly the reasons for the choice of the CF1 mouse, together with data bearing on the selection of age and weight ranges and sex are discussed elsewhere (2). For the final standard test a weight range of 16 to 18 grams was adopted.

In vitro Test

Drugs to be tested *in vivo* are selected on the basis of *in vitro* activity against *M. tuberculosis* var. bovis strain BCG, except where other considerations would lead to the belief that a drug might be effective *in vivo*.

The culture is maintained and passed weekly in the following medium:

Na_2HPO_4	3.0 Gm.
KH_2PO_4	4.0 Gm.
$MgSO_4$	0.6 Gm.
Sodium citrate	2.5 Gm.
Asparagine	5.0 Gm.

[1] From the Division of Microbiology, The Squibb Institute for Medical Research, New Brunswick, New Jersey.

Iron ammonium citrate	0.05	Gm.
0.05 per cent solution of Tween 80	0.5	ml.
Glycerine	20	ml.
Distilled water	1,000	ml.

The medium is sterilized at 15 pounds for fifteen minutes in lots of 100 ml. per flask. To each flask is added 0.4 ml. of 25 per cent bovine serum albumin fraction V sterilized by filtration. The flasks contain a 1½ inch finishing nail sealed in a glass tube as a stirrer. Ten ml. of a refrigerated culture not over 30 days old are used as inoculum. The flasks are incubated for 96 hours at 37°C. and stirred three times daily for a period of approximately one minute on a magnetic stirrer.

A control for purity of culture is performed by subculturing 0.5 ml. from each flask into a tube of beef heart infusion broth.

For the test, the above medium, but without the iron ammonium citrate and with only 0.05 per cent serum albumin fraction V, is used. Two ml. of the 96 hour culture described above are added per 100 ml. of test medium.

Substances to be tested are used at twofold dilutions. Two ml. volumes are used and the maximal concentration of drug is 0.6 mg. per ml. if the substance is freely soluble. Tests are incubated for 112 hours at 37°C. in a well humidified incubator. A standard compound 1-hydroxy-2(1H)-pyridone of known activity (3, 4) is included in every test. Results are read on the basis of turbidity (growth) in the tubes at the end of incubation. If a sharp end point is obtained with no turbidity in one tube and heavy growth comparable to that in the controls in the next tube in sequence, the inhibiting concentration is recorded as interpolated between these two tubes. When a tube of weak growth separates heavy and zero turbidity, it itself is taken as the end point. Results are reported in terms of minimal inhibiting concentrations.

Toxicity Test

Before testing a drug against the infection with tubercle bacilli in the mouse, an approximate idea of its toxicity (acceptability) is obtained. Four mice are used per dose of drug and each drug is tested at two levels, usually at a fourfold difference, until the maximal acceptable level is determined. Acceptability is judged by weight change in the individual mice. All mice are young 16 to 18 Gm. animals still actively growing. Failure to gain weight, or actual loss, is an indication of toxicity or unpalatability of the diet, or both. On first assay in the diet, one per cent is the maximum concentration used. The drug is intimately mixed with pulverized Rockland Farm mouse pellets and placed in special stainless steel food cups (4). The food cups are weighed daily and a record kept of the food consumed per 4 mice. When diet toxicities are being investigated the test runs for four days.

Parenteral toxicity is determined by subcutaneous injection. Groups of 4 mice each are used and the two levels tested (in one injection daily) are at a fourfold difference. The injections are continued for seven days and the weight curves of the individual mice followed during that period. The maximal level allowing normal weight gains is thus determined.

In vivo Test

In the infection, test drugs are administered either intimately mixed in the diet or by subcutaneous injection once daily. Two levels are usually tested at a fourfold difference. All treatments are for twenty-one days. For treatment, mice weighing 16 to 18 gm. are in groups of 10 in one cage. Twenty untreated but infected control mice in two cages are included in each test. The mice are placed on the diets forty-eight hours prior to infection. When treatment is by subcutaneous injection, the first such injection is made directly after infection. All mice are infected by the injection of 0.5 ml. of the standard inoculum (1) into the tail vein.

The average weight of each group of 10 mice is determined daily for 12 days and every

other day thereafter. The special food cups (4) are filled to 350 Gm. gross weight every day during the treatment period and the daily food consumption recorded. Autopsies are performed on all mice that die. Information is obtained and recorded on average survival time, per cent survival for 35 days, and t_{50}. The t_{50} is the calculated survival time of 50 per cent of the mice. The figure is obtained by plotting on arithmetic-probability (probit) paper (2).

Care of Personnel

All persons concerned with the infection of mice wear protective goggles. The infected mice are housed in a room liberally equipped with Westinghouse Sterilamps WL-782, style No. 1121051-A. Individuals about to work on the program receive a tuberculin test before starting work. If this is negative, they are offered BCG vaccination. All those actively engaged in the program have a complete physical examination together with a roentgenogram of the chest every six months. No cases of laboratory infection have occurred so far.

RESULTS

In vitro tests: Although the results of the *in vitro* test outlined above are used as a guide to selection of drugs for test *in vivo*, the shortcomings of the test and the possible errors involved by such a selection are fully recognized. Since twofold steps are employed, and only enough replicates run to establish that the end points obtained are of the right order of magnitude, no differences between compounds of less than fourfold are to be considered as significant. Moreover, as will develop when the results with chemical series of compounds are discussed in later papers, although the *in vitro* results may be used directly as to an indication of choice within a given series, they are of woefully little significance in selection between compounds of different series. This is to be expected. The *in vitro* results signify only the activity of the given compound when brought into contact with the tubercle bacillus in the test tube and under the conditions of the test. In the mammalian body many other factors come into play such as absorption and distribution in an unchanged or, at least, still active form, penetration to the site of infection, and maintenance at such a site in sufficient concentration and for sufficient time. Limiting factors such as systemic or local toxicity also play a part.

The validity of using a bovine organism for both the *in vitro* and the *in vivo* studies in a search for a chemotherapeutic drug active in human infection might be questioned. At least in the United States, the overwhelming majority of cases are due to the human variety of *M. tuberculosis*. The *in vitro* sensitivity of *M. tuberculosis* var. bovis strain BCG and *M. tuberculosis* var. humanis strain H37 Rv have been compared against a series of compounds. The ratio of activity of the drugs against BCG as contrasted with that against H37 Rv was remarkably constant considering the low statistical significance of the actual figures from which these ratios were obtained (see above). In general the ratio of drug required to inhibit BCG as compared to H37 Rv was 0.47, *i.e.*, BCG was from two to three times more sensitive. In 88 per cent, the ratio lay between 0.22 and 0.8. Only 4 per cent of compounds were of equal activity on both organisms or of greater activity against H37 Rv. These figures indicate that results which are not grossly misleading, as far as the ultimate goal of human therapy is con-

cerned, are likely to be obtained by using BCG. Slightly more activity will be suggested by the results than is perhaps entirely justified. No active compounds are likely to be missed. The benefits from use of BCG are a more rapid test, and safety of a considerable number of semi-skilled technicians carrying out the *in vitro* tests.

Toxicity tests: As has already been indicated, the toxicity tests on the drug given in the diet, but not those on the drug injected subcutaneously, should be regarded as tests of both acceptability (or palatability) and toxicity, or of either, rather than of toxicity alone. Objection might be raised to the small number of mice used in the diet tests for toxicity and the short period of observation time. These objections are recognized but have been overruled in favor of keeping the amount of drug required for first evaluation of worth at as small a quantity as possible, *i.e.*, from 18 to 20 Gm.

In practice it was found that 16 per cent of the time the higher drug concentration fed in the actual test was not fully acceptable (or toxic) on the basis of quantity of food eaten during the first seven days after infection (see below), but in only 1.6 per cent of the cases was it not fully acceptable (or toxic) at both levels when the difference in level wasfour fold. The acceptability test, therefore, was an adequate guide to the diet levels to be employed in the *in vivo* test, the prime purpose for which it was used.

In vivo results: As pointed out above, information is obtained on the average survival time of the mice, the t_{50} (calculated time of death of 50 per cent of the mice), the per cent surviving 35 days, and the averaged curve of weight response. In addition, autopsies are performed on all mice dying. The latter is for purposes of certification as to cause of death only. No attempt has been made to develop a score system of lesions such as is used so extensively in this and other infections to determine the norm and departures from this norm. In our opinion nothing is learned, for the purposes of the present test, by this subjective method of calculation which is not given better by the other, completely objective, methods of calculation. Such a conclusion, however, in no way argues against the obvious value of complete pathological examinations carried out to investigate the activity of any promising drug which emerges from the screen at present under consideration.

The data on the weight response have not been found particularly useful in the quantitative estimation of degree of activity. Certain useful data have, however, emerged. As will be discussed in more detail below, for many reasons tests 1 to 13 inclusive have not been considered in the overall evaluation of the results and only tests from 14 onwards have been used. One indication of the change that occurred between tests 13 and 14 is shown in figure 1. As will be seen in this figure, which shows the averaged weight response of the control untreated mice, the striking feature of the response curve of the early tests is the lack of significant gain in weight in the early part of the curve or of rapid loss in weight later. Surprisingly perhaps, the weight curves of the control mice in the three tests in the series 14 to 52, which were not entirely satisfactory, also show this lack of early weight gain. As can also be seen in figure 1, an active drug, in this

case p-aminosalicylic acid (PAS) at 0.75 per cent in the diet, shows a curve of weight response which by the tenth day has departed sufficiently from the curve of the untreated mice to be significant. Thus it is possible by watching the weight curves to foretell in many instances as early as the tenth day after infection whether an unknown drug is going to show activity or not. Such a sign of activity is only qualitative, however, and in our experience cannot be quantitated.

As indicated, some attention has been paid to survival for 35 days or longer. In 800 control untreated mice, 2 (.25 per cent) survived, one for 35 days and one for 36 days. Both were in tests in which the control group was otherwise unsatisfactory. On the other hand, some of the active compounds give significant survival at 35 days, *i.e.*, 14 days after drug administration was discontinued. Thus with Promin at 1.0 per cent in the diet, 19 out of 110, or 17.3 per cent, sur-

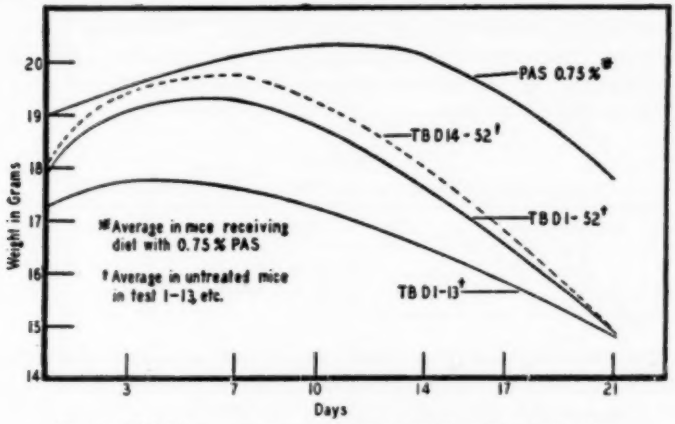

FIG. 1. Weight response curves in untreated and treated mice.

vived 35 days or longer and one animal survived 56 days. At 0.5 per cent in the diet, 6 of 60, or 10 per cent, survived. With PAS, 7 of 80 (7.8 per cent) survived 35 days at 1.0 per cent diet, 12 of 310 (3.9 per cent) at 0.75 per cent diet, and one of 300 (.33 per cent) at 0.375 per cent diet. With streptomycin at a single subcutaneous dose of between 6,000 units and 22,500 units per Kg. per day, 18 of 80 survived and, with dihydrostreptomycin at the same levels, 22 out of 90 survived. In the case of these antibiotics, survival to 60 days and beyond was not unusual. Two things, therefore, are clear. With active drugs such as the four discussed above significant survival beyond 35 days does occur. The conditions of the test are so severe, however, that only reasonably active compounds and reasonably high doses of such compounds give such survival and this criterion, therefore, is of little use in a preliminary screening program such as the one at present under consideration.

Finally consideration has been given to both the mean survival time and the t_{50} (calculated survival time of 50 per cent of the mice). The figures for the

control untreated group are shown in table 1. The results are broken down into the probable error of those from 1 to 13 and of those from 14 to 52 (the latter being current at the time of writing). As has already been indicated above, certain changes occurred between tests 13 and 14. One was that a change in the shipping and preinfection care of the mice was adopted and the other that a change was made in the preparation of the infecting inoculum.

In tests through 13, culture continuously transferred at weekly intervals was used. Such weekly transfer resulted in a gradual fall in virulence. As soon as this change was detected, only cultures of previously proved virulence were used. The change in preinfection handling of mice also seemed to have an effect. Thus the per cent rate of nonspecific deaths dropped from 4.1, which it was for the 2,320 mice used in tests 1 to 13, to 1.1 per cent for the 7,460 mice in tests 14 to 52. Whether due to one or the other of the two changes outlined, a marked difference was noted in the untreated groups of infected mice. The change in weight response has been noted above (see figure 1). The change in the t_{50} and the mean survival time with the satisfactory decrease in the probable error of a single experiment is noted in table 1.

TABLE 1

Probable Error of Mean Survival Time and t_{50} of Control Mice

	TEST 1 TO 13	TEST 14 TO 52
T_{50}	20.45 ± 2.29 days	19.86 ± 0.72 days
Mean survival time	21.89 ± 3.17 days	20.44 ± 0.73 days

It is clear from table 1 that the results are at least as satisfactory with the t_{50} as with the mean survival time. Since the t_{50} can be read sooner than the mean survival time, and also, since the t_{50} allows of less error being introduced, particularly in the treated groups by reason of the undue survival of one mouse, the t_{50} has been selected as the most satisfactory objective criterion by which to record and judge the results.

Because of the above, it has seemed justifiable to consider only experiments subsequent to test 13 as representing the established and satisfactory technique and only these have been and will be considered in the evaluation of active compounds.

Active drugs: P-aminosalicylic acid is the compound which, on the basis of early comparative evaluation in this test, has been selected as the standard active compound for oral administration. More data are available on it than on any other compound. It was soon found that, despite statements to the contrary, this drug is highly active against infection of mice with bovine strains of *M. tuberculosis*. Table 2 and figure 2 give the information obtained on the activity of PAS. On the whole, the reproducibility of both drug intake and response in t_{50} is remarkably uniform and PAS is obviously suited to serve as a standard active drug. According to the data obtained, moreover, the activity of the drug, as indicated by the t_{50}, appears as a straight line when plotted on arithmetic

paper from an intake of 2.77 to one of 12.77 M moles per Kg. per day. Below an intake of 2.77 M moles, the activity falls off rapidly.

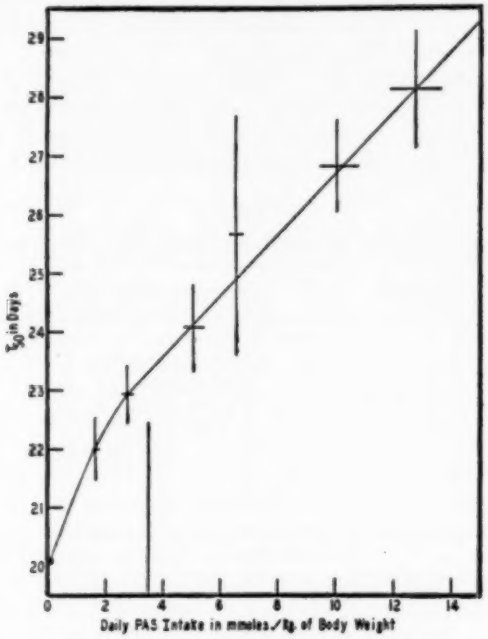

FIG. 2. Dose response curve for PAS.

TABLE 2

Drug Intake and Activity of Various Diet Concentrations of PAS, together with Probable Error (PE) and Mean Error of the Mean (ME)

CONCENTRATION IN DIET	NUMBER OF TESTS	DRUG INTAKE/DAY/M MOLES PER KG.	T_{50} IN DAYS
per cent			
0.125	4	1.67 ± 0.09 PE ± 0.04 ME	22.0 ± 0.55 d. PE ± 0.27 d. ME
0.2	4	2.77 ± 0.18 PE ± 0.09 ME	22.92 ± 0.5 d. PE ± 0.25 d. ME
0.25	4	3.51 ± 0.06 PE ± 0.03 ME	20.08 ± 2.38 d. PE ± 1.19 d. ME
0.375	30	5.06 ± 0.34 PE ± 0.06 ME	24.06 ± 0.74 d. PE ± 0.13 d. ME
0.5	4	6.54 ± 0.23 PE ± 0.11 ME	25.65 ± 2.05 d. PE ± 1.02 d. ME
0.75	31	10.08 ± 0.66 PE ± 0.12 ME	26.81 ± 0.79 d. PE ± 0.14 d. ME
1.0	8	12.77 ± 0.89 PE ± 0.32 ME	28.11 ± 1.0 d. PE ± 0.35 d. ME

Promin was also included on several occasions in tests 14 through 52. Thus a diet concentration of 0.063 per cent was tested four times, 0.125 per cent once, 0.5 per cent six times, and 1.0 per cent eleven times. The results are shown in ta-

ble 3. The number of replicates run is probably not sufficient to construct any significant response curve. All that can be said is that there is no evidence from the data at present at hand that the response curves for Promin and for PAS have a similar slope, a point of considerable importance in considering the results obtained with these and other compounds.

Two drugs, streptomycin and dihydrostreptomycin, were run as standard drugs for daily subcutaneous therapy. The results obtained are shown in table 4. It will be noted that the responses with the two antibiotics are almost identical. Here again the number of replicates are small and may be insufficient for

TABLE 3

T_{10} Response of Promin at Different Levels of Drug Intake by Mouth

CONCENTRATION IN DIET	NUMBER OF TESTS	MEAN DRUG INTAKE PER DAY	MEAN T_{10}
per cent			
0.063	4	0.16 M moles/Kg.	21.23
0.125	1	0.32 M moles/Kg.	21.8
0.5	6	1.37 M moles/Kg.	25.65
1.0	11	2.70 M moles/Kg.	27.65

TABLE 4

T_{10} Response of Streptomycin and Dihydrostreptomycin at Different Levels of Drug Intake per Subcutaneous Injection

DRUG INTAKE M MOLES/KG.	STREPTOMYCIN		DIHYDROSTREPTOMYCIN	
	Number of tests	Mean T_{10}	Number of tests	Mean T_{10}
0.0076	—	—	1	25.2
0.0091	2	26.8	2	26.2
0.0173	2	27.5	2	27.9
0.026	2	30.2	2	29.5
0.039	2	32.5	2	33.4

the construction of response curves. However, there is no evidence that the slope of the response curves for these two antibiotics would resemble that for PAS.

It has thus been possible to measure in the standardized test with the Ravenel strain and the CF1 mouse the response of drugs such as streptomycin, dihydrostreptomycin, or p-aminosalicylic acid of known antituberculous activity in man. The response which has been obtained with these compounds in the murine infection is such as to encourage one to believe that results roughly parallel to those to be expected in man can be obtained by the use of this test. At least the parallelism of the response to that seen in man would appear to be as satisfactory as any that can be claimed for those tests using the guinea pig or the rabbit.

DISCUSSION

The results obtained with this tuberculous infection in mice are remarkably uniform and reproducible whether one considers the behavior of the untreated controls or the response to treatment with active drugs. It is appreciated that this *in vivo* test is highly dissimilar to the natural infection in man or in any other animal, but it is submitted that this is of comparatively little importance, particularly at a level of investigation where search is being made for active drugs or series of drugs. When such active drugs emerge, a different type of test can be used merely by changing the conditions of the strain of mouse, strain of organism, infecting dose, and so forth. As was pointed out elsewhere, the infection in the mouse can be made to conform to almost any pattern desired, from a rapidly fatal septicemia without many lesions to a chronic fibrocaseous disease.

Actually, the test is no more dissimilar to its natural counterpart than countless dozens of other chemotherapeutic tests (with organisms producing either acute or chronic infections) which are employed daily and on the results of which definite decisions as to human or veterinary usefulness are reached. Such decisions are usually confirmed on clinical trial. There is certainly no reason, other than custom and reiteration, to believe that the tuberculous infection which employs the guinea pig is more satisfactory in indicating human (or veterinary) usefulness. Moreover, no one can doubt that this latter animal is less satisfactory for many reasons, such as higher initial and continuing expense, lack of homozygosity of available strains, and larger amounts of drug required.

With the test as outlined above, a purely objective criterion, the t_{50}, is used in evaluating the results, and any possibility of errors due to the personal factor is eliminated. Subjective criteria involving scoring based on a personal evaluation of the observed lesions would seem to be open to many objections and surely should not be considered if a satisfactory objective criterion is available. It is believed that the above presentation shows that satisfactory objective criteria are available and that of these the t_{50} is the best.

As has already been pointed out, both gross and microscopic pathological data find their true usefulness in the evaluation of any active compound which emerges, and will be thoroughly explored under such circumstances.

SUMMARY

Information is presented on the results obtained by the use of certain standardized procedures for the evaluation of antituberculous compounds *in vitro* and *in vivo*.

The use of a homozygous mouse (the CF1) and a bovine strain of *M. tuberculosis* (Ravenel) has allowed the development of a test which shows good reproducibility both in the untreated infected mice and in the mice treated with known active drugs. Small numbers of mice are used and the results can be evaluated in under four weeks. Compounds of unknown antituberculous potential are now being tested by the above methods and the results will be presented later.

SUMARIO

Empleo del Ratón en una Prueba Normalizada de la Actividad Antituberculosa de los Compuestos de Origen Natural Sintético. III. La Prueba Normalizada

La información presentada abarca los resultados obtenidos con el empleo de ciertos procedimientos normalizados para la valuación de los compuestos antituberculosos *in vitro* e *in vivo*.

El empleo de un ratón homócigo (el CF1) y de una cepa bovina del *M. tuberculosis* (Ravenel) ha permitido elaborar una prueba dotada de buena reproducibilidad en los ratones, tanto infectados y sin tratar como tratados con drogas activas conocidas. El número de ratones usados es pequeño y el resultado puede ser apreciado en menos de cuatro semanas. Con las técnicas descritas se hallan ahora en comprobación compuestos de desconocida actividad antituberculosa, y el resultado se presentará después.

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THE USE OF BIOMETRIC METHODS IN COMPARISON OF ACID-FAST ALLERGENS

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In allergen studies, comparisons of potency are of great importance. They have been a matter of concern to U. S. Department of Agriculture workers on tuberculosis and related infections in cattle.

Johnson and associates (3) have published studies on multiple testing of allergens on sensitized cattle, measuring reactions by skin thickening. During 1945 and 1946 some trials of technique were made and the results were analyzed. The work was done in cooperation with Dr. H. W. Johnson, Dr. A. H. Groth, and Dr. A. B. Larsen of the U. S. Bureau of Animal Industry's Regional Animal Disease Research Laboratory, Auburn, Alabama, and Dr. L. A. Baisden and Mr. R. R. Henley of the Washington office of the Pathological Division of the Bureau. The same methods of analysis have been found applicable to skin reactions on guinea pigs, measuring reaction by diameter or area of skin affected. The writer (6), has written a discussion of applications of experimental design to these problems.

BASIS OF COMPARISONS

The use of biometric methods is not so complex as is often believed. The basic reason for thinking one material is better than another is that it consistently gives better results in repeated trials. Statistical analysis simply gives mathematical expression to the consistency or lack of consistency. A measure of variability familiar to many investigators is the standard deviation. In present day work the *variance*, the square of the standard deviation, is much used, since it is more convenient arithmetically and since variances from different sources are additive. By the use of the technique known as *analysis of variance* (described by Snedecor (5)), variances from different sources may be separated and compared when the experimental plan permits it. Care in planning an experiment is necessary to permit a separation of all pertinent effects.

In comparing the potencies of preparations by their biological reactions, the basis of comparison is the ratio of concentrations required for a given effect. A material affording a particular result with only one-tenth as high a concentration as another material must be ten times as potent as the other material. In present day work, the reactions are usually compared by analysis of variance and related to the concentrations by a curve. Interpolation on the curve gives the best estimate of concentration required for a given reaction. A carefully planned experiment will permit several short cuts. These methods are discussed in numerous articles by Bliss and associates. A particularly specific and useful treatment is by Bliss and Marks (1). This sort of comparison is termed biological assay.

In analysis of the trials of technique of testing tuberculins on cattle, it was found that there was little variation in the operators' inoculations and measure-

ments, or between the two sides of an animal. There were marked differences in reaction between cows, and also differences between regions of the body. A good experimental plan will balance out these differences by giving materials equal representation on all the areas and the animals used.

Varying concentrations of a material showed marked differences. A long series of dilutions, amply replicated, was tried out and the results were studied. Dilutions ranged from 1:20 to 1:1,536,000 O. T.[1] The relation of concentration and reaction was not linear. However, the logarithms of concentration plotted against reactions gave a nearly linear curve, though it appeared faintly S-shaped

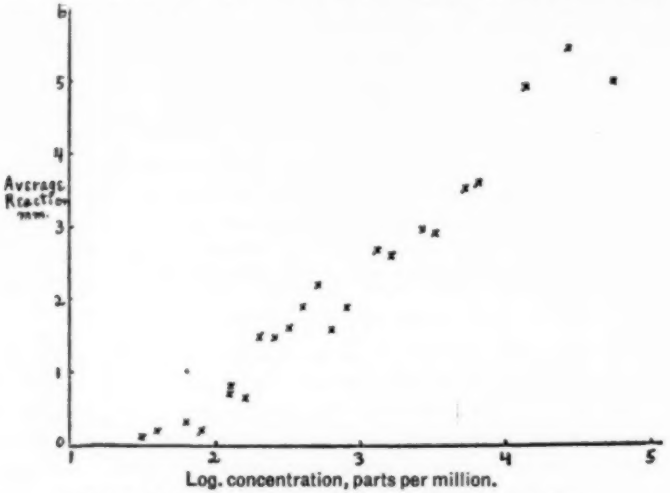

FIG. 1. Relation between log concentration and reaction

Typical results, large series of concentrations; 4 cows, 12 sites per concentration, tuberculin precipitate on para-sensitization. (Site differences cause a wider scatter than should be found, but trend still shows up.)

(figure 1). At lower concentrations the slope was less steep. Linearity was good, however, over a wide range of medium and high strengths. The linearity of reaction with log concentration was used in analysis. Heterologous materials gave curves less steep than homologous materials. For example, tuberculin on tuberculin-sensitized cattle gave a steeper curve than johnin on such animals. The two types of curves were well separated at high concentrations and seemed to converge at lower ones. A rather high level of reactions seems desirable in comparing allergens.

EXAMPLE OF TYPICAL DATA

As an example, a test of a johnin (30-I-3) against its silicotungstic acid precipitate on five paratuberculosis-sensitized cows is selected. One side of each

[1] O. T. = Old Tuberculin, as originally prepared by Koch.

cow was injected with the parent material, the other with the precipitate, each in a number of corresponding sites. The sites on each side were divided into 14 groups of 3 adjacent sites each. Each group of three received three concentrations (1:40, 1:400, 1:4,000 O. T.), one on each site. Reactions to the six treatments (two materials at three concentrations) are averaged for each cow. Although the 14 units of a treatment on each cow were on different areas, varying in reaction, the units of each treatment covered the same areas. Hence, the averages are comparable.

ANALYSIS OF VARIANCE

The analysis of variance must first separate variance for cows, for treatments, and for residual error. In this experimental plan, the error variance is that for

TABLE 1

Average Reactions in Millimeters of Increased Skin Thickness in Test of Johnin vs. its Precipitate

COW NUMBER	PARENT MATERIAL			PRECIPITATE			TOTALS
	1:40	1:400	1:4,000	1:40	1:400	1:4,000	
816	10.1	4.5	1.5	12.0	7.4	3.1	38.6
817	8.0	5.0	2.2	9.9	6.5	3.5	35.1
818	10.5	5.4	2.5	15.2	7.9	3.7	45.2
819	12.6	5.4	2.6	13.4	7.9	4.3	46.2
820	6.6	2.5	1.2	7.7	4.3	1.6	23.9
Totals	47.8	22.8	10.0	58.2	34.0	16.2	189.0
Mean	9.56	4.56	2.00	11.64	6.80	3.24	

The totals are given merely for convenience in analysis; they would not normally be entered in the table.

interaction, or differential effect of cow and treatment. If treatments behave differently on some cows than others, the error will be high and will indicate that there is doubt that treatments are really different. The error variance is really a measure of how much treatments might vary, if they were not in fact different at all.

Calculation is carried out by convenient short cut methods. The variance is the square of the standard deviation, and methods for calculation are similar. First must be calculated the total *sum of squares of deviations* of all items from the general mean. This sum is then subdivided according to sources of variation. The portions are divided by the proper divisors ("degrees of freedom," one less than the total number). The quotients are the "mean squares" or *variances* expressing variation due to each source. The variances may be compared by a standard test (Snedecor, cited above).

To get the total sum of squares of deviations, all items are squared and the squares totaled. From this total is subtracted a "correction factor," which is the sum of the items squared and divided by their number. This subtraction

reduces the sum of squares of original items to the sum of squares of *deviations* from the mean. In our material the calculation is:

$$[(10.1)^2 + (4.5)^2 + \text{et cetera} + (4.3)^2 + (1.6)^2] - (189.0)^2/30$$

or 1618.76 minus 1190.70, which is 428.06.

The portion of this sum of squares due to cow variation is quickly computed from cow totals by another short cut formula: $[(38.6)^2 + \cdots + (23.9)^2]/6 -$ 1190.70, or 54.41. (The divisor is 6, because 6 items enter each total.) The portion due to treatment variation is estimated:

$$[(47.8)^2 + \cdots + (16.2)^2]/5 - 1190.70 \text{ or } 351.37.$$

The portion due to differential effect or experimental error is conveniently estimated by subtracting the others from the total. (It could be estimated directly by more complex methods.) It is: 428.06 − 54.41 − 351.37, or 22.28. A summary of the analysis to this point may be seen in table 2.

TABLE 2

Summary of Analysis of Variance

SOURCE OF VARIATION	NUMBER OF ITEMS IN GROUPS	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Total	30	29	428.06	—
Cows	5	4	54.41	13.60
Treatments	6	5	351.37	70.27
Error	—	20	22.28	1.11

The ratio of "cow" or "treatment" mean square to "error" mean square may be compared with the tabular "F" value (Snedecor (5, ch. 10)). Both are very significant.

REFINEMENTS IN ANALYSIS

Further study is necessary to define the important effect of treatments. It is easy to see that nearly all the effect is due to difference between concentrations, and that it is doubtful how much is due to materials. The sum of squares between treatments could be subdivided, using an extension of methods already shown. The special arrangement of the material, however, makes possible a study by easier methods which are well adapted to such problems. This is the method of separation of sums of squares for individual degrees of freedom discussed by Snedecor (5, ch. 15). Bliss and Marks (cited above) give the special application to the present problem.

It is noted above that reactions show a nearly linear relation with logarithms of concentration. The three concentrations (1:40, 1:400, 1:4,000) are more clearly stated as parts per 10,000 (250, 25, and 2.5). The logarithms of these are 2.4, 1.4, and 0.4 respectively. This equal spacing of logarithms makes possible a special application of single degrees, to separate the part of the sum of squares due to linear relation, or regression, with logarithms of concentrations. Other parts of interest may also be separated.

In the single degree method there are several general rules as to arrangement of comparisons, divisors, et cetera. The arrangement suited to this problem is presented in table 3.

TABLE 3

Single Degree Analysis of Treatments (Data of table 1)

	MATERIAL AND LOG CONCENTRATION (PARTS PER 10,000)						NET SUM	DIVISOR	SUM OF SQUARES = (NET SUM)²/Divisor
	Parent			Precipitate					
	2.4	1.4	0.4	2.4	1.4	0.4			
	Reactions, sum								
	47.8	22.8	10.0	58.2	34.0	16.2			
Material	+1	+1	+1	−1	−1	−1	−27.8	30	25.76
Regression (linear)	+1	0	−1	+1	0	−1	+79.8	20	318.40
Non-parallelism	+1	0	−1	−1	0	+1	− 4.2	20	0.88
Curvature	+1	−2	+1	+1	−2	+1	+18.6	60	5.77
Opposed curvature	+1	−2	+1	−1	+2	−1	+ 5.8	60	0.56

It will be noted that these sums of squares add to the previous total for "treatments" in table 2 (351.37).

The sums of squares for each degree of freedom is obtained by taking the net sum indicated by the signs, squaring it, and dividing by the correct divisor. The divisor is the sum of squares of the coefficients, multiplied by the number of replications. For example, for "curvature," the net sum is: $[(+1)\ (47.8)] + [(-2)\ (22.8)] + [(+1)\ (10.0)] + [(+1)\ (58.2)] + [(-2)\ (34.0)] + [(+1)\ (16.2)]$, which equals + 18.6. The divisor is $5\ [(+1)^2 + (-2)^2 + (+1)^2 + (+1)^2 + (-2)^2 + (+1)^2)]$, or 60. The sum of squares is $(+18.6)^2/60$, or 5.77.

These sums of squares are also mean squares because each is based on only one degree of freedom. As mean squares, they may be compared to the general error variance, 1.11, in the F test. This test shows the difference between materials to be quite significant; the variance due to linear regression is outstandingly important; variance due to curvature is barely significant and of small importance. The linear relation accounts for most of the trend. The other two variances are nonsignificant; parallelism of trends in the two materials is satisfactory, and no trace of "opposed curvature" appears.

It has been noted that this simple analysis could not be carried out if concentrations were not equally spaced on the log scale used. With unequal spacing, certain single degrees of freedom could be defined, but they could not be directly identified with linear regression, curvature, et cetera. Also, the part of the variation due to linear trend could be evaluated, but it would have to be done by general methods, fitting a line and taking deviations from it (Snedecor (5, ch. 6)).

MEASURING POTENCY

The analysis shows that materials are really different, by comparison of their variance with the error variance. To measure the *extent* of the difference, further analysis is needed. The comparison of concentrations needed for a given effect

is the vital one. These concentrations can be estimated from curves showing relation of concentration and reactions. The linear relation with logarithms of concentration makes it simple to estimate the *difference* in logarithms, and later express this as a *ratio* of concentrations. The line expressing this relation is called a regression line. The fact that regressions are practically parallel for the two materials makes the distance between the lines a measure of the difference desired. (If they were not parallel a statement of difference could be made only for a given level of response.)

Regressions could be fitted directly, and estimates of log difference made by general methods. However, there have been developed short cut methods, if experimental arrangement makes possible the analysis of table 3 and if nonparallelism and curvature are not important. Bliss and Marks (cited above) illustrate these convenient methods. The difference in logarithms is estimated as $\bar{C}_2 - \bar{C}_1 + (K\ I\ D)/B$; where $\bar{C}_2$ and $\bar{C}_1$ are average concentrations of the test and standard substances, I is the interval between logarithms of concentration, K is a constant depending on number of concentrations used, D is the square root of variance between materials, and B the square root of the variance for linear regression. Here $\bar{C}_2$ and $\bar{C}_1$ are equal and I is simply 1, so that the expression boils down to KD/B. For three concentrations, K is $\sqrt{8/3}$ or 1.63; for two concentrations, 1; for four concentrations, $\sqrt{5}$. Thus our estimate of log difference is $(1.63\ \sqrt{25.76})/\sqrt{318.40}$. This is 8.28/17.84, or 0.464. It may be stated as +0.464 in evaluating the potency of the precipitate compared to its parent material. A reversal of the test would change the sign. The antilogarithm of 0.464 is 2.91. We estimate that 2.91 times as much parent material as precipitate is required for the same effect. Hence, the precipitate has a potency estimated as 2.91 times that of the parent material.

ERROR OF POTENCY DETERMINATION

Finally, it is desirable to estimate the standard error of the potency ratio. This may be readily done in terms of logarithms. A short cut formula (Bliss and Marks) is available for solutions of the type used above. The standard error is $(s/b)\ \sqrt{2/N}$; where s is the standard deviation, square root of the error variance (here 1.11); b is the average regression coefficient of reaction on log concentration; N is the total number of trials of each material (here 15). A more complex formula is used where $\bar{C}_1$ and $\bar{C}_2$ are unequal. To use the error formula, we must estimate the average regression coefficient. It can be calculated in this simple analysis as the quotient of "net sum" over "divisor," but a more general method is given. The coefficient for each material can be quickly calculated from logs of concentration and average associated reactions. It is the sum of products of deviations of these two variables, divided by the sum of squares of deviations of logs of concentration. The sum of square of deviations of logs is quickly calculated: $(0.4)^2 + (1.4)^2 + (2.4)^2 - (0.4 + 1.4 + 2.4)^2/3$, or 2.00. In this particular case this might be easily calculated by taking deviations directly from the mean, 1.4; squaring and summing. The sum of products mentioned, for the parent material, is: $(9.56 \times 2.4) + (4.56 \times 1.4) + (2.00 \times 0.4) - (16.12 \times 4.2)/3$, or

+7.56. (16.12 is the sum of the mean reactions; 4.2, the sum of logs.) The regression coefficient is then + 7.56/2.00, or +3.78. This is an estimate of slope of the line; for every advance of 1.0 in log concentration, an advance of 3.78 mm. in reaction is expected. The regression coefficient for the precipitate is estimated as +4.20. The average regression coefficient is +3.99, which can be used as "b" in the formula; s will be $\sqrt{1.11}$, or about 1.054; $\sqrt{2/N}$ will be $\sqrt{2/15}$ or 0.365. Thus $(s/b)\sqrt{2/N}$ will equal 0.096.

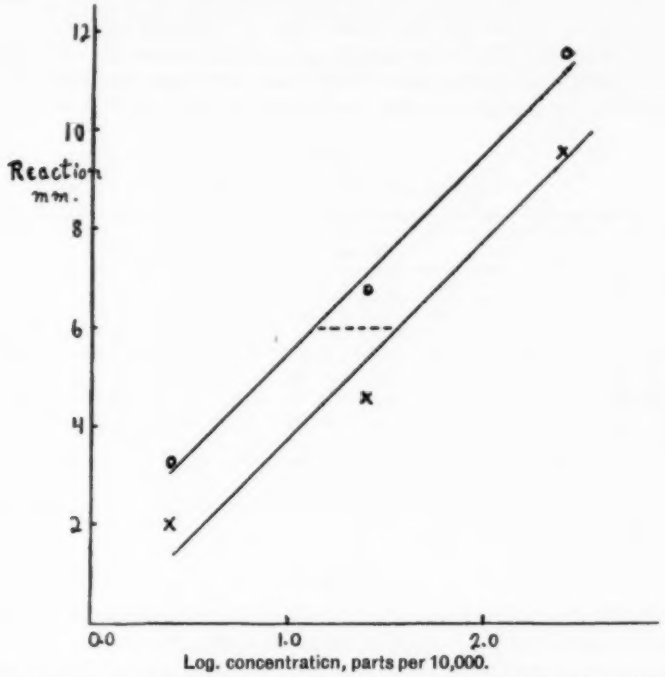

FIG. 2. Average regression fitted to reactions of parent material (X) and precipitate (O) from table 1.

Dotted line shows log. potency ratio, graphic determination, as near 0.45.

The log of the ratio is thus estimated as 0.464 with a standard error of 0.096. In figure 2, the mean reactions are plotted against logs of concentration, with the average regression fitted and the distance between the lines measuring the log of potency ratio.

EXAMPLE OF WORK WITH GUINEA PIGS

Guinea pigs are frequently used in preliminary evaluations of tuberculins; they often afford a quick and convenient trial (See McIntosh and Konst (4); a letter

from Greene (2) in England also discusses the work further). When the same materials have been tested on both cattle and guinea pigs, rankings have been similar. Results have appeared to be a little less consistent with guinea pigs than with cattle.

Reaction has been measured by size of skin area showing irritation; areas, or average diameters, of the round or oval spots have been used. It seems that linear measurements are more likely to be "normal" and suited for analysis than areas. In the one comparison of the two criteria made in this work, diameters were a little more precise than areas; this question needs more study.

Table 4 shows results of a comparison of 3 B.A.I. tuberculins on each of 4 mammalian-sensitized guinea pigs. Three concentrations were used, thus employing 9 sites per animal; complete randomization was used.

TABLE 4

Comparison of Tuberculins on Guinea Pigs

(Average diameter of irritated spots in millimeters, 24 hour reading)

PIG NUMBER	TUBERCULIN NUMBER AND CONCENTRATION								
	876			880			881		
	1:100	1:500	1:2,500	1:100	1:500	1:2,500	1:100	1:500	1:2,500
39,152	16.00	13.00	9.00	16.25	10.00	11.25	17.50	11.00	8.25
38,068	15.50	12.00	10.25	16.25	10.50	9.50	15.75	14.25	9.00
38,062	25.00	12.00	11.00	14.25	15.50	11.25	19.50	13.50	8.25
38,075	14.75	13.00	12.00	17.50	10.50	10.00	17.50	15.25	9.25

Following the same methods of analysis as in tables 2 and 3, and comparing tuberculins 880 and 881 with 876 as a standard, logs of potency ratios were 0.20 and 0.07, respectively. Standard errors of logs of potency ratios were 0.18 and 0.16; thus, no real difference was found in the materials.

SUMMARY

The application of biometric methods to the problem of comparison of allergens is discussed and an example is presented using results from cattle in detail. A second example of work with guinea pigs is more briefly cited. The results show that biometric methods are not difficult; that they measure consistency and give common sense results; and that experiments must be carefully planned to make full use of biometric aids in interpretation.

SUMARIO

El Empleo de las Técnicas Biométricas en la Comparación de los Alergenos Acidorresistentes

Discutida la aplicación de las técnicas biométricas al problema del estudio comparativo de los alergenos, preséntanse ejemplos de los principios comprendidos, usando los resultados obtenidos en bovinos y cobayos. El resultado demuestra que las técnicas biométricas miden la constancia y que hay que pla-

near cuidadosamente los experimentos para aprovechar debidamente los auxiliares biométricos en la interpretación.

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EDITORIAL

Tuberculosis in Medical Teaching

Convincing arguments can be advanced to support the thesis that tuberculosis is the most important among the diseases which are both preventable and curable. To readers of this journal, the reasons do not require iteration why this disease remains one of the paramount medical problems of this century, despite the considerable decline in its mortality in some countries. The importance of the problem is indeed conceded by most leaders in medicine and medical education, and the necessity to instruct students and young physicians in this field is widely recognized. Yet there is, in this country at least, and particularly among physicians who have devoted themselves to the study and treatment of the disease, a growing concern about the adequacy of professional training as it relates to tuberculosis. This concern has manifested itself within the American Trudeau Society by the formation of a Committee on Medical Education. The investigations made and the conferences held by this committee have undoubtedly aided in clarifying the problems, in discovering present deficiencies, and in pointing the way to remedial solutions.

It requires no special investigations, however, to recognize the principal cause of present day inadequacies in undergraduate and postgraduate medical training in tuberculosis. The cause is at once apparent in the relative isolation of this field of medical activity from the teaching centers. The sanatorium movement, which at the opening of the twentieth century was numerically still in its small beginnings, has in the last fifty years succeeded in segregating in special institutions nearly all of the patients under active hospital treatment for pulmonary tuberculosis. Desirable as this may be in other respects, it has created special problems in medical education.

The tuberculosis patients who might serve for clinical instruction have, for the most part, been withdrawn from the teaching hospitals. Consequently, neither the students nor their principal teachers have sufficient opportunity for direct observation and study of this prevalent disease. In many teaching hospitals, pulmonary tuberculosis patients are not admitted or they are rigidly segregated in a special division which serves more as an isolation and transfer depot than as a unit for definitive medical care. Usually such units, like the special tuberculosis hospitals and sanatoriums, are staffed separately from the general medical services. The physicians who undertake the care of these patients are often too overburdened to take as active a part in the general medical work as their colleagues in other branches of internal medicine. Thus a subspecialty of internal medicine has arisen which, though by no means narrow, since it has grown to include pulmonary diseases in general, is further separated in practice from general medicine than are most of the other recognized subspecialties. The other internists, for their part, have small opportunity, and often little interest, to share in the responsibility of caring for the tuberculous

sick. The lack of interest is particularly conspicuous in academic circles and in teaching hospitals, in many of which indeed the attitude appears to be that tuberculosis is scarcely a concern of the internist. In most of the American medical colleges this condition has existed not only through many generations of medical students, but through several professorial dynasties.

In theory, the special status, which the care of tuberculous patients has come to occupy because of the real or assumed necessity for segregation, need not be detrimental to medical education. Courses for undergraduates can be arranged in sanatoriums and tuberculosis hospitals. Affiliations can be provided for the exchange of interns and residents between such hospitals and general hospitals, or between general services and tuberculosis services in general hospitals. Some such arrangements are in fact widely prevalent and it is not the lack of them which constitutes the problem. There is, to be sure, great variation in the quality of instruction and in the amount of time devoted to these courses and training periods in different centers. The particular success achieved in the competition for curriculum time depends in large part on local factors. Neither is the main problem the training of tuberculosis specialists or even of teachers. This must also depend on the organization of tuberculosis facilities in particular communities.

The larger problem is whether the segregation of tuberculous patients, to the extent to which it exists, is necessary and whether the relative isolation of this field of medical activity is greater than it need be in the interest of good care and of scientific progress. Considerations of climate, which initially influenced the selection of remote localities for the establishment of tuberculosis institutions, have been largely discarded. In recent years the infectious nature of the disease and its chronicity have been the main factors in the continued segregation of patients. The latter considerations are still valid and the provision of special facilities is necessary, though these need not be remote. The progress of the last several decades in thoracic surgery and, to some extent also, the more recent developments in chemotherapy, have made the provision of complete hospital facilities essential, either within the tuberculosis institutions themselves or easily accessible. The skills required in the modern treatment of pulmonary tuberculosis are many and varied. The frequent association of tuberculous and nontuberculous complications adds further to the need for practically all medical and surgical specialty services, not excluding research facilities. The closest possible association and interchange of information and ideas between the tuberculosis and general hospitals is for these reasons evidently desirable. Particularly is it desirable for the teaching hospitals, which are the principal centers of clinical research, to maintain active contact with tuberculosis institutions, and even to provide a quota of beds for the interchange of patients.

The admission of tuberculosis patients to general teaching hospitals on a more liberal basis than has become the custom would do more than any other measure to improve medical education in tuberculosis. In a teaching hospital the mere presence of a tuberculosis section, even if limited to 25 or 50 beds, is of educational value. It permits a greater integration of instruction in this subject with instruc-

tion in general medicine and surgery. More importantly, it promotes a better understanding of the disease on the part of the general medical and surgical attending and teaching staff, and a greater awareness of its clinical and research problems. No less importantly, it permits the physicians with a special interest in tuberculosis and pulmonary diseases to pursue the sub-specialty without losing close contact with general medicine. By actual participation in the work of the general services they may not only extend the breadth of their own medical knowledge, but also influence greatly the attitudes of their teaching colleagues.

In the last analysis, it is not what students learn in special courses, but what they absorb from their principal teachers, which forms education. As long as the complacent attitude prevails in the general services that a case of tuberculosis is primarily a disposition problem, so long will the education of students and interns be deficient. Only exceptional circumstances, such as contraction of the disease itself, will readily turn the young physician's interest to a closer study after the conditioning he usually receives in his clinical clerkship and internship.

To the phthisiologists and teachers of tuberculosis, the remedies should be clear. No amount of propaganda about the pre-eminent importance of tuberculosis as a world medical problem is of the slightest value. Neither is insistence on a particular amount of curriculum time desirable or necessary, beyond a reasonable minimum which is not likely to be denied. Rather it is essential to attempt in every way possible to reduce the barriers, both psychological and practical, which have grown up between this sub-specialty and general medicine. By the very nature of the problem, these barriers cannot all be removed nor should special experience and skill in the clinical problems of tuberculosis be surrendered. Greater participation by pulmonary specialists in general medical service and teaching can, however, be developed without sacrifice of special competence. They need not acquiesce in over-specialization if they make it a condition of their service to maintain opportunities for pursuing broader interests. By so doing they can surely improve their own teaching and, not improbably, they may awaken in their colleagues an interest in reciprocal participation.

As tuberculosis, in some aspect, is the concern of every practitioner in whatever specialty, so its teaching is the responsibility of the entire medical faculty. The phthisiologist's concern is with the segment of the problem which lies within the field of internal medicine. As the thoracic surgeon is primarily a surgeon, so the phthisiologist is primarily an internist. The more he can participate with other internists in joint clinical research and teaching enterprises the better will be the education of the students who are under their mutual guidance.

CARL MUSCHENHEIM

LETTER TO THE EDITORS

TUBERCULOSTATIC ACTIVITY OF AUREOMYCIN IN VITRO AND IN VIVO

To the Editors of the American Review of Tuberculosis:

The letter of Steenken and Wolinsky[1] on the activity of aureomycin against the tubercle bacillus prompts a brief note as to the results in our hands. The growth inhibiting action on H37Rv, Ravenel, and a BCG strain of *M. tuberculosis* has been tested *in vitro*. Using Kirchner's medium, to which has been added 0.03 per cent of Triton A-20[2], and reading the test at the end of 5 days, a sharp end point of inhibition has been obtained at 4.55 γ per ml. for H37Rv, 12.5 γ per ml. for Ravenel and 0.36 γ per ml. for BCG in the absence of serum. With 5 per cent sheep serum in the same medium at the end of 5 days, a range of partial inhibition of BCG occurred from 0.27 γ to 1.0 γ per ml.

The maximal tolerated daily dose of aureomycin *per os* for the CF1 albino mouse weighing approximately 18 Gm. was determined and found to be 5 mg. Groups of 10 mice each of the same CF1 strain weighing approximately 18 Gm. were then infected with the Ravenel strain of *M. tuberculosis* var. bovis by the standard method[3]. Two groups of 10 mice each were left as untreated controls, one group received 2 mg. of aureomycin *per os* daily and one group 0.5 mg. *per os* daily. As a positive control *p*-aminosalicylic acid incorporated in the diet was used in one group at 0.75 per cent and in the other at 0.375 per cent. As has been shown elsewhere[3], the delay in the t_{50} (calculated time of survival of 50 per cent of the mice) has been shown to be a highly satisfactory, reproducible, and objective method of measuring antituberculous activity. Under the above circumstances, neither dose of aureomycin had any effect on the t_{50}, whereas *p*-aminosalicylic acid in the same experiment gave a delay of 7.2 days at the 0.75 per cent level and a delay of 4.5 days at the 0.375 per cent level. Although on a per kilogram basis the 2 mg. daily dose represents approximately thirty-five times the dose given by Steenken and Wolinsky, it will be noted that even at this dose aureomycin *per os* had no detectable effect upon the course of infection with *M. tuberculosis* in the mouse.

THE DIVISION OF MICROBIOLOGY
THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH
NEW BRUNSWICK, NEW JERSEY.
APRIL 22, 1949

G. RAKE
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[1] Steenken, W., Jr., and Wolinsky, E., Am. Rev. Tuberc., 1949, *59*, 221.

[2] Subsurface active agent incorporated in the medium at the suggestion of Dr. R. Dubos of the Rockefeller Institute for Medical Research.

[3] Rake, G., *et al.*, Am. Rev. Tuberc., 1949, *60*, 121.

BOOKS

R. A. Willis: *Pathology of Tumors. Pp. xxiii, 992. Butterworth & Co., Ltd., London, England; The C. V. Mosby Company, St. Louis, Mo., 1948. Price $20.00.*

Doctor Willis' approach to the problem of the pathology of tumors is at the same time highly individual and honestly objective. His personal bias gives spice to the book. He has combined his own long and wide experience in the study of neoplasm with his broad knowledge of the writings and conclusions of other students of the field, but he has never let the latter sway his own judgment. He never defers to authority nor straddles a difference of opinion and yet his confidence in the correctness of his own views falls short of the didactic in almost every instance.

The volume is divided into two parts. The first, which comprises about a fifth of the whole, is concerned with a general discussion of neoplasia. It is well done and at the same time regrettably concise. It might well have been expanded into a full companion volume for the main body of the work. This Part I disposes quickly with the enigma of definition, logically discounts and discards all classifications but that one proposed by the author, touches the salients of the experimental production of tumors, deplores the defects of statistical analysis, briefly catalogues tumors of other species than man, and goes on to a discussion of growth, spread, metastasis, and the general nature of neoplasia. The condensation of this large body of knowledge and experimental endeavor into little more than two hundred pages is a feat remarkably and admirably contrived. From it a reader who is quite unfamiliar with the field is able to acquire a general background that is valuable either for itself alone or for more specific and detailed literary inquiry into special aspects of cancer study. As such it is of value and interest to students of medicine whether undergraduate or postgraduate.

Part II comprises the remaining four-fifths of the book. It is entirely unlike Part I in that its concern is a detailed and systematic description of the morphology, behavior, and incidence of the many individual tumors that arise in the mammalian body. It is a textbook for the advanced study of neoplasia and for the practicing surgical pathologist, and a reference book for the clinician who day by day encounters the distressingly common or the puzzlingly rare new growths.

One by one Doctor Willis considers in systematic detail the epithelial tumors of the organs or organ systems of the human body. The approach to each is much the same, and this is a praiseworthy uniformity which adds to the value of the book both as a reference work and as a pedagogical instrument. An example of this is the treatment of the "Epithelial Tumours of the Trachea, Bronchi, and Lung." Here, after a brief and semihistorical introductory paragraph dealing chiefly with pulmonary carcinoma comes a section on "Frequency, Age, Sex, Race and Species Incidence" which after a categorical discussion is followed by "Possible Causative Factors" again considered in detail and in turn followed by

the more morphological aspects of the lesions, "Site of Origin", "Growth, Complication and Local Spread", and "Metastasis." Bronchial adenomas and other benign tumors are treated in a sufficient manner at the end of the chapter.

The adherence to this pattern of treatment of the various anatomical areas and their tumors tends unavoidably to monotony but this defect is offset by the facility to reference afforded by it. The profusion of case histories of patients or specimens studied personally by the author, which he uses constantly throughout the text to illustrate his thesis, adds greatly to the individuality of the work and comfortingly confirms the reader in his opinion that he is perusing something by someone who has "been there." The interspersion of these case histories softens the effect of the reiterated personal pronoun, especially in the author's expression of opinion.

The text is copiously and excellently illustrated with photomicrographs of rare tumors and of the less common aspects of familiar tumors. Doctor Willis quite properly has not attempted to duplicate standard textbook reproductions.

The bibliography at the end of each chapter is not only full and timely but has the almost unique virtue of presenting in bold face type those works which the author has thought for one good reason or another to be of outstanding value, and to each he has appended in parentheses his reason for so thinking. Thus discrimination is added to completeness.

JOHN M. PEARCE
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VICENTE SANCHIS-OLMOS: *Skeletal Tuberculosis. Pp. xiii, 261. Williams & Wilkins Co., Baltimore, Md., 1948. Price $5.00.*

According to the preface, this book has been written "with the intention of emphasizing that which is new or little known." It is written by a Spanish orthopedic surgeon and is based on his experience and teaching in Spain, supplemented by observations made while he was in Boston as a graduate student in orthopedic surgery. The author reviews the pathogenesis, the pathology, the roentgenologic examination, symptoms and their evolution, and the treatment. A second part deals with special subjects.

In discussing pathogenesis and pathology, the author goes into considerable detail about various types of tuberculous lesions, such as granular, exudative, and caseous synovitis and osteo-arthritis. These undoubtedly exist, and the author may be correct in his statements about these and their practical significance. Nevertheless this reviewer believes that the author fails to show convincing proof, in some of the roentgenograms exhibited as demonstrating granular osteitis and exudative synovitis, that they are such.

There are actually some statements to which exception may be taken, such as: "In other instances [spondylitis] it is the deformity [kyphosis] that first attracts attention." This is unfortunately true at times, but we believe that the diagnosis of tubercular spondylitis should be made before the deformity develops. Again, in discussing pathologic dislocations, the author mentions two classes,

early and late. The early one is said to be due to "a large intra-articular effusion that hastens the loss." Such a dislocation in a tubercular joint without some tissue destruction seems to the reviewer a very remote possibility.

The book is valuable as a compilation of known facts regarding the incidence of tuberculous osseous lesions in a well-known institution in Spain. As a basis for teaching the more theoretic phases of pathology and pathogenesis, it is worth while if it is realized that a considerable part of that portion of the book must be based on theory.

R. K. GHORMLEY

RENÉ SAND, Professeur de Médecine Sociale à l'Université libre de Bruxelles: *Vers la Médecine Sociale. Pp. 671, J.-B. Bailliere et Fils, Paris, 1948.*

From the point of view, historical-bibliographical rather than analytical-pathological, this book is a most valuable enrichment of present day literature on social medicine. The wide scope of the materials covered is overwhelming, and the author's bibliographic knowledge is evidenced in each chapter starting as a rule with presentation and quotation from ancient history throughout the ages until most recent time. Already the list of contents gives proof of it. In individual chapters are treated: the history of the medical professions; the history of hospitals; and the history of personal hygiene. All of these and the subsequent accounts are accompanied by numerous quotations from Graeco-Roman, Chinese, Indian, Arabic, Middle Age, and Modern sources. These are followed by chapters dealing with the history of public hygiene, epidemics, and public health, and the history of social hygiene. The latter is divided into a good many subsections. For example, on the first programs of social hygiene throughout the world, the role that single inspiring personalities played in the development of public health and city sanitation, such as Philippe Buchez in France, Salomon Neumann and Rudolf Virchow in Prussia, Max von Pettenkofer in Bavaria, is reviewed. Subsequent sections in this long chapter deal with the coming of preventive medicine, marriage counseling, the care of pregnant mothers and their new born, school hygiene, physical health and periodical examinations of school children, and the fight against tuberculosis and other mass diseases. Moreover such subjects as housing and urbanization, physical and hygienic education, and many more items of modern preventive medicine are covered. Further chapters treat the history of factory work since antiquity, the protection of labor, legislation on work conditions and occupational injuries, the history of public assistance and social insurance, the history of the sciences of man, among which statistics, demography, anthropometry, social psychology, genetics, eugenics, and population politics are named and briefly annotated. Finally, the book closes with a longer chapter considering in some detail, country by country, the present advancement of social medicine, its recent developments, achievements, and institutions. A relatively large subsection in this chapter is devoted to social medicine in the English-speaking world, especially Great Britain and the United States, after the earlier developments in Germany and

France have been reported upon at some length. A few sections in this chapter also deal with the still controversial definitions, limitations, and methods of social medicine, and lastly with the teaching and curriculums of this new branch at various universities and medical schools.

There certainly is a tremendous field of medical science and its relationship to social conditions covered in the book, and it is almost past belief that a single personality, even of such erudition and age-old experience as René Sand in various brackets of hygiene and public health, is able to master this vast field. Small wonder, then, that certain subjects, such as the control of tuberculosis, the fight against venereal diseases in its singular stages, the detection of cancer and its differential mortality, the explanations and applications of vital statistics, the importance of anthropometric evaluations, et cetera, are only sketchily dealt with. Nevertheless, the wealth of bibliographic footnotes is amazing everywhere. There are almost no statistical tables in the large book to characterize differential prevalence of diseases in different socio-economic or occupational groups. Evidently, as the title of the book, *Towards Social Medicine*, may indicate, the author's intention was to describe the trend of public health and group medicine since antiquity rather than to give a systematic social pathology at the present time by demonstrating the degree to which diseases are influenced (or not) by social-environmental conditions. The latter can be done only by careful presentation and analysis of pertinent statistics; the former (the trend of social medicine throughout history) is masterfully elucidated and amply supported by an abundance of bibliographic footnotes and appendix notes which in themselves make the book worth possessing as a source for further inquiries in the field of social medicine. There is at the end a supplementary bibliography on general subjects, an alphabetic index of names, and a carefully planned and useful index by countries and subjects treated in the book.

GEORGE WOLFF
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A. BRADFORD HILL: *Principles of Medical Statistics. Fourth edition. London, The Lancet Limited, 1948. Pp. xi and 252. Price 7s. 6d.*

This is the fourth (revised and enlarged) edition of the "Principles" after the third edition was reprinted three times during the war without essential changes, so great was the demand among medical circles for this little book on statistical treatment of medical problems. This in itself is a high recommendation of the book from the practical point of veiw. I have before me the first edition that appeared in 1937 and may well be in a position to compare the two as to their practical value. The book has increased from 171 pages in the first edition to 252 in the present without having lost its principal character of being a readable introduction to all who take time out to study a few fundamental statistical problems. A new chapter (The Average) has been added to demonstrate how central values (mean, median, mode) can be easily calculated from grouped figures (by using arbitrary units) without involving arithmetical hardships.

Such a chapter has a highly educational value for all who shrink from statistical computations. The further chapters on problems of sampling (Proportions, Differences, Discussion of the Chi-square test) have been retained for the larger part as they were printed in the former editions with some supplementary examples added. Special attention is given to the discussion of the statistical significance of differences which are of great importance in clinical research, as well as in public health work, to prove (or disprove), for instance, the value of a new therapeutic agent, the efficacy of a vaccination procedure, the variation of body measurements between groups of school children, et cetera. There are two chapters on the calculation, use, and misuse of the correlation coefficient, similarly as in the first edition. The chapter on life tables, which was somewhat sketchy in the former editions, has been essentially enlarged and illustrated by examples, emphasising the great importance of survival rates and other life table values (life expectancy) in vital statistics and population theory. Of special value, it seems to the reviewer, are the following three chapters on fallacies and difficulties in the use of vital statistics. Here it may be mentioned that the so-called proportional death rates (proportion of specific deaths among all deaths) are critically viewed. These ratios are too often used (because they are readily obtainable) instead of the death rates proper in a population at risk. Tuberculosis is chosen as an example to show that such proportion (though arithmetically correct) is never a good measure for the risk of dying from tuberculosis; for it depends just as much on the relative frequency of *other* causes of death, the increase or decrease of which must necessarily influence the proportion of tuberculosis deaths. The same criticism is applied to the use or misuse of crude and standardized death rates; two new chapters are added to discuss the standardization methods usually employed. It is obvious that the death rate in a chosen standard population is a fictitious rate for the risk in a given population, but it will be of great value as a comparative index in populations of different age and sex composition and, in the United States in particular, of different race composition. In such cases, the overall (or crude) rate gives a distorted picture. The direct and indirect methods of standardization are shown in special examples.

There is no doubt that Hill's "Principles," though somewhat more technical in the new edition, will gain more friends in medical circles. There are quite a few mathematical-statistical textbooks crowded with formulas and derivations from abstract examples on the market, but there is hardly a better guide available to the public health worker and physician for statistics applied to medical problems. The work is critical without being sophisticated. In his introductory chapter the author states quite ably "No statistical method can compensate for a badly planned experiment;" in public health, however, we have to deal as a rule "with statistics that come from no deliberate experiment but that arise, and are collected, from a population living and dying in an everyday course of events." To analyze these "mass experiments," which cannot be planned a priori, a good deal of knowledge in the subject matter is necessary, in addition to statistical methods, to make the statistical analysis a live contribution to research.

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THE AMERICAN REVIEW OF TUBERCULOSIS ABSTRACTS

Edited by LAWRENCE B. HOBSON

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CLINICAL STUDIES, TUBERCULOSIS

Dangers of Vitamin D_2 Therapy.—Although previous good results warrant the use of large doses of vitamin D_2 in certain forms of tuberculosis, this treatment is not without danger. Pathologically the lesions produced by this treatment are identical with those seen after the administration of irradiated ergosterol. There is calcification of the large vessels of the lungs, the heart, the dura, the digestive tract, and of the kidneys. The damage is particularly severe in the kidneys where calcifications have been observed in the renal epithelium, the interstitial tissue, the glomeruli, and the arterioles. Clinically, intoxication is usually manifested by gastro-intestinal symptoms but it is noteworthy that severe kidney damage may precede clinical signs. This is especially true in kidneys which have been damaged by tuberculosis. Elevation of the blood urea nitrogen is an early danger signal and is observed in most patients with renal tuberculosis. This azotemia usually persists after the treatment is stopped. Elevation of the blood calcium and phosphorus is observed less frequently. The dosage of vitamin D_2 is 15 mg. once or twice a week and is continued as long as it is tolerated. This is rarely more than five or six weeks in patients with renal tuberculosis.—*Des accidents de la vitamino-thérapie D_2 a fortes doses, en particulier dans la tuberculose rénale, M. Secrétan, Schweiz. med. Wchnschr., January 24, 1948, 78: 49.*—(*H. Marcus*)

Pneumothorax for Minimal Tuberculosis.—Statistics from a French sanatorium for high school and college students between the ages of 15 and 20 show that an increasing number of the cases are being diagnosed by routine examination; 20 per cent in 1945 and 48 per cent in 1947. During 1947, only 14 per cent of the patients had far advanced tuberculosis; most had well localized, small infiltrations with or without cavitation. Of the 145 patients discharged from the sanatorium since its opening in 1945, 121 had been treated with pneumothorax and 81 per cent of these had favorable results. Only 57 per cent of the 33 patients treated with a simple sanatorium regimen responded favorably. The maximum observation period for any case was 2.5 years. There were 40 asymptomatic patients with minimal lesions on the roentgenogram but without tubercle bacilli in the sputum. Sixteen of them were treated with pneumothorax and 24 with rest alone. After 30 months, 8 relapses had occurred in the conservatively treated group. None of the 16 patients with pneumothorax relapsed during that period and all were able to resume an increasingly active mode of life.—*À propos des indications du pneumothorax dans les formes benignes de la tuberculose pulmonaire, (une statistique du sanatorium des lycéens et collégiens a Neuf-Moutiers-en-Brie), J. Lardanchet, Rev. de la tuberc., 1948, 12: 845.*—(*V. Leites*)

Extrafascial Pneumothorax.—Cavities which have remained open under a thoracoplasty can be collapsed by an extrafascial pneumothorax. An extrafascial pneumothorax also has the advantage of preventing paradoxical breathing which is frequently seen after thoracoplasty. In the 20 patients reported by the author an extrafascial pneumothorax was established following a second stage thoracoplasty.—*Die extrafasziale Luftfüllung nach*

Obergeschossplastik, K. Janauschek, Wien. klin. Wchnschr., December 24, 1948, 60: 825.—(G. C. Leiner)

Decortication and Lobectomy.—The results of thorocoplasty have been disappointing when pulmonary tuberculosis was uncontrolled by pneumothorax and complicated by tuberculous empyema or an unexpandable lung. The Schede or Keller operations with resection of the parietal pleura have been the best available surgical treatment in the past. However, the incidence of cavity closure and healing of the empyema has been low and the degree of mutilation and disfiguration of the patient often severe with these procedures. A new, combined procedure is suggested for selected cases. It consists of lobectomy when the tuberculosis is confined to one lobe with decortication of the remaining lobe or lobes. Two illustrative cases of upper lobe tuberculosis, uncontrolled by pneumothorax, are presented. In neither case could the lung be re-expanded and in neither case was it thought that thorocoplasty over the unsuccessful pneumothorax would produce a satisfactory collapse. One case was further complicated by a mild tuberculous empyema. Each was treated successfully by lobectomy with extrapleural dissection and decortication of the remaining lung tissue. In both instances streptomycin and penicillin were given parenterally for one week before and for three weeks following the operation. Lobectomy, with decortication if necessary, should be considered when a pneumothorax has been unsuccessful in obtaining cavity closure despite a seemingly adequate anatomical collapse of the diseased lobe which re-expands slowly. This would avoid the risk of spreading the disease during a prolonged period of re-expansion before thoracoplasty.—*Decortication and lobectomy for uncontrolled pulmonary tuberculosis with tuberculous empyema or unexpandable lung, I. A. Sarot, Quart. Bull., Sea View Hosp., April, 1948, 10: 47.—(K. T. Bird)*

Hemothorax after Pneumonolysis.—It is recommended that the blood be removed from a hemothorax which has developed following a pneumonolysis. This was done by thoracotomy in 11 cases. The operation should be performed within eight to fourteen days after the pneumonolysis, as soon as the temperature has become normal.—*Die operative Ausräumung der vollgebluteten Pneumolyse, K. Janauschek, Wien. klin. Wchnschr., October 22, 1948, 60: 680.—(G. C. Leiner)*

Clinical Studies, Nontuberculous Diseases

Boeck's Sarcoid.—The pathological observations in 4 cases of Boeck's disease showed lesions which resembled one another in many respects in the acute as well as in the healing stages despite the varied clinical courses. Although complete regression may occur, fibrosis and hyalinization without calcification were seen in all 4 cases. In the acute stage there was a predominance of histiocytes occurring in discrete tubercles without many lymphocytes and without necrosis. In the healing and healed stages fibrosis, a tendency toward hyalinization within uncalcified tubercles, and the occasional retention of outlines of the tubercles in hyalinized areas, are suggestive of Boeck's disease.—*Boeck's disease, Rosenthal, J., & Feigin, I., Arch. Path., June, 1948, 45: 681.—(E. Bogen)*

Paradoxical Mediastinal Swing in Pleurisy.—The occurrence of mediastinal shifting or Holzknecht-Jacobson's sign in some cases of bronchial obstruction is well known. It has been reported occasionally as the sole indication of an obstruction later demonstrated by bronchoscopy. The phenomenon consists of a shifting of the mediastinum toward the affected side during inspiration, particularly deep inspiration, and a return to or beyond its former position during expiration, especially following a cough. The respiratory immobility of the affected lung, which neither completely fills on inspiration nor is completely emptied on expiration, produces the shifting of the mediastinum. The expanded sound lung exerts pressure and the incompletely filled hemithorax on the affected side acts as

an attracting force. Atelectasis or emphysema are not necessarily factors. In certain cases of pleurisy, the authors have noted a paradoxical mediastinal shifting toward the sound side, and report 4 cases in which it occurred. In all of them there was complete immobility of the affected side of the thorax including the diaphragm and the shifting occurred when the sound lung emptied rapidly following a cough.—*Le balancement médiastinal de sens paradoxal dans certaines symphyses pleurales et le signe d' Holzknecht-Jacobson, M. Levrat & G. Despierres, Le Poumon, July-August, 1948, 4: 209.*—(*A. T. Laird*)

Pedicles of Upper Lobes.—The anatomical relations of the arteries, veins, and bronchus which make up the stem of the upper lobe of the lung are of especial interest to the surgeon during a lobectomy. According to the newer technique the use of the tourniquet has been abandoned, the vessels are ligated separately, and the bronchus is closed by suturing. The authors report anatomical studies made on the cadaver and during the course of the removal of 16 upper lobes. The pedicles of the right and left upper lobe differ because of the presence of the lingula on the left and the passage of the left branch of the pulmonary artery around the left upper lobe bronchus. The right upper lobe bronchus usually arises from the outer wall of the main bronchus about 21 mm. from the tracheal bifurcation. Anomalies in its origin are comparatively rare though it has been found to start directly from the trachea or to leave the main bronchus in a common trunk with the branch to the middle lobe. The course of the bronchus is outward and upward to the upper lobe, where it may terminate in 2, 3 or 4 branches. The upper lobe of the right lung always receives several arterial branches including the principal or anterior artery, the accessory or retrobronchial artery and, rather frequently, other important branches. These may not be seen unless the pedicle is examined by way of the interlobar fissure. The most important of the veins is the posterior vein of the upper lobe. In the left lung, the upper lobe bronchus comes off the main bronchus farther from the bifurcation of the trachea than on the right side. It is often not more than 15 mm. in length but is considered long enough to suture. The bronchus terminates in an ascending and a descending branch. The left main branch of the pulmonary artery is smaller in caliber than the right and the arrangement of its subdivisions is variable. Approach by way of the interlobar fissure is necessary to locate some of them. The left superior pulmonary vein consists entirely of branches derived from the upper lobe. Variations in the origin of arterial and venous branches occur on both sides.—*Les pédicules des lobes pulmonaires supérieurs, A. Maurer & F. Tobé, Le Poumon, January-February, 1948, 4: 7.*—(*A. T. Laird*)

Technique of Upper Lobe Lobectomy.—This paper is a discussion of the removal of the upper lobe by dissection and severing of the elements of the pedicle as practiced by the authors in a series of 16 lobectomies. Their series included removal of the right upper lobe in 8 cases, of the right upper and middle lobe in 3, and of the left upper lobe in 5. They were undertaken for an abscess of the lung in 9 cases, for cysts of the lung in 5 cases, and once each for cancer, tuberculosis, and bronchiectasis. The feature of the operation to which the authors call special attention is the ligature of each of the blood vessels in the pedicle with the closure of the bronchus by sutures. The patient was placed in the lateral position and general anesthesia, usually cyclopropane-oxygen-curare, with tracheal intubation was employed. The incision was made along the fifth rib or in the fifth interspace; in adults the fifth rib was removed by subperiosteal resection. The principal artery and the veins of the right upper lobe were reached from the front. The other arteries of the right upper lobe were approached by way of the interlobar fissure which in some cases had to be reconstituted. The bronchus was finally attacked from the fissure or from its posterior aspect. In most cases it could

be sutured directly without preliminary closure of any branches. The method of Sweet was followed, the bronchus being clamped on the pulmonary side and then sectioned and sutured progressively. Pleuralization of the bronchial stump on the right side was accomplished by utilizing the posterior mediastinal pleura which was made to cover the anterior surface of the bronchus. In the left lung the aortic pleura was used. It was always necessary, especially in cases of abscess or tuberculosis, to prevent the escape of secretions from the upper lobe bronchus before the bronchus was sutured. Otherwise they might have been aspirated into the other lung or into the lower lobes of the same lung. The authors preferred previous preparation of the patient, tracheal aspiration and minimal pulmonary manipulation rather than the use of an obturator or clamp. Aseptic lesions without bleeding pleural surfaces do not require drainage but infected cases may need long-continued aspiration. Of the 16 patients who were operated upon, 3 have died: one of tuberculous meningitis after the removal of an upper lobe for tuberculosis and 2 of pulmonary abscesses. In 4 cases a bronchial fistula developed.—*Technique de la lobectomie supérieure par dissection des éléments du pédicule, A. Maurer & J. Mathey, Le Poumon, January–February, 1948, 4: 21.*—(*A. T. Laird*)

Relative Safety of Upper Lobe Lobectomy.—A study of 63 cases in which an upper lobe of the lung was removed shows that the results of this operation are as satisfactory as those following other pulmonary resections. This series, operated on at the Massachusetts General Hospital in Boston from 1943 to 1946, included 45 cases in which the upper lobe alone was removed, 18 in which the upper lobe and some other portion of the lung were resected, 16 in which the middle lobe alone was removed, one in which the apical portion of the lower lobe, and one in which the middle lobe, and a portion of the lower lobe were removed. In the series there were 13 cases of lung abscess, 21 of tuberculosis, 12 of cancer, 10 of bronchiectasis, 3 of pulmonary cysts, 2 of chronic pneumonia, and 2 of benign tumors. In all of the cases, the blood vessels in the pedicle of the lobe were ligated separately, the bronchus was divided and sutured, and the stump was pleuralized. Prophylactic penicillin therapy was given for several days before the operation; transfusions and plasma infusions were given during the procedure. Drainage was employed after resections for tumors or suppurative lesions; of the 63 cases, 45 were drained. Lobectomies for abscess or tuberculosis are attended by considerable risk as are those in febrile patients and in cases where more than one lobe must be removed. The conditions of the pleura, the amount of expectoration, and the necessity for drainage also influence the outcome. The prognosis is improved by the use of adequate preoperative preparation of the patient, simple operative procedures with as little trauma as possible, excellent anesthesia, periodic re-expansion of the lung during the operation, careful closure of the bronchial stump, airtight drainage, and careful postoperative care. In this series of 63 resections of an upper lobe, there was immediate re-expansion of the remaining lobes and an uneventful convalescence in 30 cases (47.6 per cent) and delayed re-expansion in 33. Among the latter, were 15 cases of pleural effusion and 18 cases of bronchial obstruction. There was no re-expansion in 8 cases (11.1 per cent) with subsequent serious complications. There were 5 deaths (8 per cent mortality). These results are comparable to those in a series of 99 resections of lower lobes performed at the same hospital during the same period. There was immediate re-expansion and prompt recovery in 41 cases (41.1 per cent). There were serious complications in 11 cases (11.1 per cent) and 5 patients died (5.1 per cent mortality). It thus seems that removal of an upper lobe of the lung is about as safe as removal of a lower lobe if the proper personnel and facilities are available.—*La lobectomie supérieure, R. H. Sweet & H. LeBrigand, Le Poumon, January–February, 1948, 4: 46.*—(*A. T. Laird*)

Bronchial Adenoma.—The clinical course of bronchial adenoma can be divided into three distinct phases: (*1*) Phase of hemoptysis. Other symptoms are lacking at this time and, because of the bleeding, a mistaken diagnosis of tuberculosis is sometimes made. (*2*) Phase of bronchial stenosis. Recurrent attacks of pneumonitis, often associated with pleural effusion, frequently lead again to a diagnosis of tuberculosis at this stage. (*3*) Phase of suppurative pneumonia and atelectasis. The most important diagnostic procedure is bronchoscopy although some help may be derived from roentgenographic studies that include bronchograms and planograms. If the patient is seen early, especially in the first phase of the illness, removal of the tumor through the bronchoscope should be attempted. This is not usually possible in the second phase, and in the third phase only lobectomy or pneumonectomy is curative. Other indications for excisional surgery are the impossibility of removing the tumor through the bronchoscope, extraordinarily great vascularity of the tumor, and severe suppurative disease with bronchiectasis or abscess formation distal to the tumor.—*Das Krankheitsbild und die Differential-diagnose des Bronchialadenoms, A. Kappert, Schweiz. med. Wchnschr., January 17, 1948, 78: 26.*—(*M. Marcus*)

Tumor Cells in Sputum.—The value of microscopic examination of the sputum for tumor cells is illustrated by 3 case reports. All 3 patients were suspected of having pulmonary tuberculosis but sputum studies revealed the correct diagnosis of primary pulmonary carcinoma.—*Apical pulmonary carcinoma and tuberculosis: The value of sputum cell study in the differential diagnosis, M. Bergman, B. A. Shatz & I. J. Flance, J. A. M. A., November 13, 1948, 138: 798.*—(*H. Abeles*)

Bronchial Washings.—Bronchial washings according to the technique of Manoel de Abreu were performed on 257 patients. Demonstration of tubercle bacilli in the specimens obtained was attempted only by culture or guinea pig inoculation. A comparison between the results of gastric and bronchial lavage was carried out on 150 cases with roentgenographic changes suspicious for tuberculosis. The patients had no expectoration or sputa without demonstrable tubercle bacilli. Bacteriological evidence of tuberculosis was found in 47.2 per cent of these cases; in 22.4 per cent by both gastric and bronchial lavage, in 20 per cent by bronchial washings only, and in 4.8 per cent by gastric washings only. In 107 persons with roentgenographic changes of undetermined origin and sputum without tubercle bacilli, bronchial washings revealed the bacilli intermittently in 41.1 per cent. Among 90 persons in whom routine roentgenograms showed apparently fibrotic changes, 35 per cent had tubercle bacilli demonstrable intermittently with this method. Bronchial washings are of special interest in determining the length of pneumothorax treatment and in evaluating the results of collapse therapy.—*La rechereche du bacille tuberculeux par le lavage pulmonaire, Ch. Gernez-Rieux, A. Breton & A. Sevin, Rev. de la tuberc., 1948, 12: 743.*—(*V. Leites*)

Sarcoidosis.—This case report traces the evolution of Boeck's sarcoid over a period of ten years to its termination in miliary tuberculosis. A 27 year old male patient was under the supervision of the tuberculosis control division of his home town for ten years. There was persistent hilar adenopathy demonstrable roentgenographically, with a negative tuberculin reaction. No biopsy was possible because of absence of peripheral lymph node enlargement. During the period of observation the patient remained in good health and was able to work. He developed miliary tuberculosis without meningitis after ten years observation. His tuberculin test became positive and his disease was rapidly fatal. Autopsy showed many hematogenous tuberculous lesions throughout the viscera. The paratracheal, paraesophageal and abdominal lymph nodes showed very extensive hyalin changes with epithelioid cells and

Langhans giant cells. The connective tissue was so dense that it replaced the normal architecture of most lymph nodes entirely. Although there was fresh caseation in some of the nodes at the bifurcation of the trachea, the nodular hyalinization of most of the lymph nodes was characteristic of long-standing sarcoidosis. In addition to these findings, there was fresh tuberculous spondylitis of the second dorsal vertebra.—*Morbus Besnier-Boech mit Uebergang in Miliartuberkulose, Jenny Müller & A. Pedrazzini, Schweiz. med. Wchnschr., February 14, 1948, 78: 126.*—(*M. Marcus*)

Antimicrobial Therapy

Streptomycin Resistance.—A series of 13 cases of pulmonary tuberculosis was treated with streptomycin. The patients ranged from 15 to 30 years of age and were suffering from acute progressive bilateral tuberculosis of recent origin. All had cavities. Streptomycin was given in doses of 0.5 Gm. every six hours intramuscularly for six months to some of the group and for four months to the remainder; the observation period was six months. All the strains of tubercle bacilli obtained prior to treatment were either half as sensitive to streptomycin as the control strain of H37Rv, twice as sensitive, or equal in sensitivity, with one exception. The bacilli from 12 of the 13 cases showed increased resistance to streptomycin after treatment and 11 of these strains were 32 or more times as resistant as the control strain. In most cases the resistance of the bacilli increased rapidly and then remained about the same. Two-thirds of the patients developed resistant bacilli within 48 days. There was a definite relation between the clinical progress before resistant organisms appeared and the degree of resistance that eventually occurred. The patients doing well in the early stages of treatment had bacilli which developed a low degree of resistance, while those patients doing poorly later produced more highly resistant organisms. The higher the degree of resistance, the more rapidly it appeared. There was no significant correlation between the mean day that resistant bacilli appeared or the degree of resistance that developed and the clinical progress of the patient after that development. Nor was there any demonstrable relation between the time at which resistant strains developed and the general condition of the patient on admission. There was no relationship between the degree of resistance and the extent of cavitation. There are two possible mechanisms by which strains of tubercle bacilli resistant to streptomycin might appear in the sputum: (*1*) the entire surviving bacterial population might become gradually adapted to growth in the presence of the drug; or (*2*) the patient's population of tubercle bacilli before treatment with streptomycin might contain organisms with varying degrees of resistance, the more highly resistant being few in number but under treatment having an advantage over the sensitive strains and gradually replacing them.—*Streptomycin resistance in pulmonary tuberculosis, J. Crofton & D. A. Mitchison, Brit. M. J., December 11, 1948, 4588: 1009.*—(*R. W. Clarke*)

Changes in Meningitis after Streptomycin.—The pathological observations in 10 children who had been treated with streptomycin for tuberculous meningitis are reviewed. The exudative process showed a tendency to organize and form gradually sclerosing scar tissue. The proliferative processes, such as miliary tubercles, were encapsulated by connective tissue; early miliary tubercles were transformed into connective tissue. Hyalinization and calcification were seen also. These changes, which are signs of healing in a pathological sense, were the causes of late complications such as internal hydrocephalus and obliterating endarteritis resulting in encephalomalacia; rare late complications were tuberculoma and lymphocytic encephalitis.—*Pathologisch-anatomische Befunde bei mit Streptomycin behandelten Fällen von Meningitis tuberculosa, H. Chiari, Wien. klin. Wchnschr., December 31, 1948, 60: 837.*—(*G. C. Leiner*)

Therapy of Meningitis in Children.—The dosage of streptomycin in the treatment of tuberculous meningitis in children has been modified in an attempt to avoid the high dosage recommended by Hinshaw and Feldman and to improve on the small and only moderately effective dosage of Cocchi. The following schedule has proved very successful: first month of treatment, 500 to 1,000 mg. of streptomycin daily intramuscularly and 1 mg. per Kg. of body weight daily intrathecally; second month, same intramuscular schedule but an intrathecal injection only every other day; third month, same intramuscular dosage with intrathecal treatment only about twice a week depending somewhat on the changes in the cerebrospinal fluid. At the same time 1.5 to 3. Gm. of Promin is given daily intravenously and once a month a massive dose of vitamins A and D is administered. Early diagnosis and early treatment are the most important conditions for success. Treatment of other bacterial forms of meningitis is also discussed in this paper.—*Der heutige Stand der Meningitis-Therapie im Kindesalter, G. Fanconi, Schweiz. med. Wchnschr., February 14, 1948, 78: 121.*—(*H. Marcus*)

Streptomycin for Tuberculous Pericarditis.—A 17 year old male Negro who had acute tuberculous pericarditis with effusion, tuberculosis of the left lower lobe, and tuberculous lymphadenitis was treated with streptomycin. The pericarditis responded very favorably to the treatment.—*Streptomycin in acute tuberculous pericarditis, a case report, H. C. Meredith, Jr., Am. Heart J., January, 1949, 37: 129.*—(*G. C. Leiner*)

Streptomycin for Miliary Tuberculosis.—Streptomycin has been used to treat children with disseminated tuberculosis. Of 9 children with miliary pulmonary tuberculosis, 5 were discharged and are well, 4 are still under treatment. Of 44 children with tuberculous meningitis, 11 were discharged and are well, 5 are ready to be discharged, 12 are still under treatment, and 16 have died. Of 2 children with miliary peritonitis, one was discharged and is well, one is being treated and is improving. Of 7 infants with tuberculosis without miliary dissemination, one died but 6 improved.—*Streptomycinbehandlung der miliaren Tuberkulose, O. Ruzicka, Wien. Klin. Wchnschr., December 31, 1948, 60: 840.*—(*G. C. Leiner*)

Fundi during Streptomycin Therapy.—The eye-grounds of 42 children were examined repeatedly during streptomycin treatment for tuberculous meningitis or miliary pulmonary tuberculosis. In more than one-third of the cases, changes of the papilla were observed; in more than one-third, changes of the chorioidea, multiple miliary tubercles, or solitary tubercles, were seen. In most cases, there was a quick regression of these foci soon after treatment with streptomycin was begun. In cases which showed slow regression of these foci, complications and relapses occurred. No evidence of any toxic effect of streptomycin on the optic nerve was seen.—*Augenhintergrundsbefunde bei der Streptomycinbehandlung der Meningitis tuberculosa unter der miliaren Lungentuberkulose, F. Meyer, Wien. klin. Wchnschr., December 31, 1948, 60: 842.*—(*G. C. Leiner*)

Otolaryngological Tuberculosis.—A group of 20 cases of laryngeal tuberculosis, associated with pulmonary tuberculosis, was treated with parenteral streptomycin. Involvement of the epiglottis, aryepiglottic folds, and arytenoids was found in 45 per cent of this group so that dysphagia and hoarseness were predominant symptoms. Streptomycin was given in a dosage of 2.0 Gm. daily for one week followed by 1.0 Gm. daily for 83 days. Blood levels frequently were 20 to 40 γ per cc. of serum during the first week; thereafter the levels were between 5 to 10 γ per cc. of serum. Upon completion of therapy, 14 showed a "healed larynx," a fibrotic end stage with no evidence of activity; 4 showed an "almost healed larynx," fibrosis, except for a small area of injection or some other slight involvement; the remaining 2 showed improvement. An average of 21 days of treatment

was required before subjective and objective evidence of improvement was noted. It required sixteen weeks for healing to occur. Of the 20 cases, 11 had vertigo at some time during treatment. Compensation with diminution or disappearance of vertigo occurred in every instance, despite an average age of 46.7 years. Streptomycin should be used for moderate or advanced tuberculosis of the larynx regardless of the status of the lungs. Nine cases of tuberculous otitis media and mastoiditis were treated with streptomycin but no definite conclusions could be drawn from this series. Some cases of tuberculous otitis media alone were healed by the use of streptomycin. In tuberculous mastoiditis, surgery is still indicated although streptomycin is a valuable adjuvant.—*Streptomycin therapy in otolaryngologic tuberculosis, J. G. Gilbert, J. S. Aronoff & D. M. Rosen, Quart. Bull., Sea View Hosp., January, 1948, 10: 3.*—(*K. T. Bird*)

Streptomycin for Tuberculosis.—Twenty-six patients with far advanced bilateral pulmonary tuberculosis, whose disease was too acute or too extensive for routine collapse measures, were treated with streptomycin. In 24 patients sufficient improvement occured to permit some form of collapse therapy. Streptomycin should never be used in a therapeutic test or during a period of observation of a patient for whom other treatment may be required. There is great danger in using streptomycin too early, since its effectiveness may be lost at a time it is most needed. Streptomycin treatment should not be instituted until a definite plan for the treatment of the patient has been worked out. In this group of patients streptomycin was used to prepare the patients for collapse therapy, that is, as an adjunct and not for complete cure.—*Streptomycin as adjunct in treatment of acute pulmonary tuberculosis, E. R. Levine, W. S. Klein & A. Forman, J. A. M. A., November 13, 1948, 138: 808.*—(*H. Abeles*)

Streptomycin Stomatitis.—Severe stomatitis was observed in 3 patients during the course of intramuscular streptomycin treatment. The stomatitis disappeared promptly after discontinuance of the streptomycin treatment.—*Stomatitis due to streptomycin, H. Beham & H. Perr, J. A. M. A., October 16, 1948, 138: 495.*—(*H. Abeles*)

Penicillin Dust Inhalation.—An apparatus is described for the use of penicillin dust for infections of the respiratory tract. Three hundred and fifty seven patients were treated with 100,000 units of penicillin dust one to three times a day. Allergic reactions were observed in 3 per cent of the patients after contact dermatitis was prevented by creaming the face. Maximum levels of penicillin in the blood were obtained one hour after inhalation. The best results were seen in the treatment of bronchiectasis.—*Inhalation of penicillin dust, L. Krasno, M. Karp & P. S. Rhoads, J. A. M. A., October 2, 1948, 138: 544.*—(*H. Abeles*)

Laboratory Studies

Sputum Concentration.—A method is described for concentrating sputum prior to its inoculation into guinea pigs. (*1*) The sputum is liquified by incubation for twenty-four hours. The vitality of the tubercle bacilli is not impaired as it is by treatment with sodium hydroxide. (*2*) After dilution with ten times its volume of distilled water, the sputum is centrifuged. (*3*) The sediment is neutralized and 6 drops of penicillin G (5,000 units per cc.) are added to control contamination. Inoculation is easy with this preparation.—*Homogénéisation des crachats et centrifugation du bacille de Koch vivant en vue de l'inoculation au cobaye, F. Tison, Ann. Inst. Pasteur, February, 1948, 74: 128.*—(*V. Leites*)

Cultures in Dubos Medium.—Dubos liquid medium was inoculated with suspensions of 0.1 to 0.01 mg. of bovine or human tubercle bacilli. Growth was observed as a sediment

on and after the fifth day. The cultures rarely produced a homogeneous clouding of the fluid. No cultures could be obtained by inoculating the medium with organs from tuberculous guinea pigs.—*Essais de culture de BK dan le milieu de Dubos, J. Valtis, F. van Deinse & J. Solomides, Ann. Inst. Pasteur, February, 1948, 74: 434.—(V. Leites)*

Laboratory Diagnosis of Tuberculosis.—No certain diagnosis of active tuberculosis can be made by roentgenography alone; tubercle bacilli must also be recovered from the patient's sputum or body fluids. Tubercle bacilli can be demonstrated more regularly by selecting caseous flecks in the sputum than by concentration methods. Small numbers of tubercle bacilli can be more readily detected by culture than by smear. Acid fast saprophytes cannot be differentiated from virulent tubercle bacilli on stained smears, but competent bacteriologists can distinguish, with considerable accuracy, pathogenic from nonpathogenic acid-fast bacilli on the Lowenstein-Jensen egg medium. In its present form the Dubos Tween-albumin liquid medium is not recommended from primary isolation. Urine and gastric contents frequently contain nonpathogenic acid fast bacteria; for this reason, guinea pig inoculation should be practiced on such specimens.—*The laboratory diagnosis of tuberculosis, M. M. Cummings, Am. J. Pub. Health, March, 1949, 39: 361.—(Max B. Lurie)*

Fluorescent Method for Smears.—A comparison was made between the results obtained with the fluorescent technique of Richards and Miller and those obtained with Pottenger's method for the Ziehl-Neelsen staining of sputum and gastric contents for tubercle bacilli. Of 280 direct smears of sputum stained by the Ziehl-Neelsen method, 148 (52.8 per cent) were found to contain acid-fast bacilli. The same number of direct smears from the same patients, when stained and examined by the fluorescent method, yielded 154 (55.0 per cent) positive smears. Concentrates of these 280 sputa yielded 186 (66.1 per cent) positive smears by the Ziehl-Neelsen method and 190 (67.8 per cent) positive examinations by the fluorescent method. In another series of 514 sputum concentrates, 347 (67.5 per cent) were found to be positive by the Ziehl-Neelsen method and 354 (68.4 per cent) by the fluorescent technique. Of 210 gastric concentrates, 89 (42.3 per cent) were positive by the Ziehl-Neelsen method and 108 (51.9 per cent) were positive by the fluorescent method. These figures show a higher percentage of positive examinations with the fluorescent technique. However, there are certain disadvantages to the use of this method. For best results a dark room is preferable. There are organisms other than *M. tuberculosis* which give fluorescence. Of 25 gastric concentrates found positive only by the fluorescent method, 11 were confirmed by guinea pig inoculation. This discrepancy is probably due to the presence of avirulent or nonviable tubercle bacilli or saprophytic acid-fast bacilli in the gastric contents and sputum. The possibility of finding acid-fast saprophytic bacilli is even greater in stools and urine. In addition, the likelihood of error in diagnosis is increased by any particles which have retained the auramine dye and so may be mistaken for bacteria. Of 63 samples of gastric contents in which one to three bacilli per slide were found after a careful search, 59 were positive when tested by inoculation. It may therefore be concluded that positive results obtained by the Ziehl-Neelsen method are of greater value than those obtained by the fluorescent technique and correlate better with the clinical condition of the patient. On the other hand, negative results by the fluorescent method are of great value. The results with this method may be improved by refinements in technique.—*La microscopía fluorescente en la investigacion del mico bacterium-tuberculosis, A Gómez Muriel, Rev. mex. de. tuberc., May-June, 1948, 9: 81.—(F. Pérez-Pina)*

Effect of Chick Embryo Extract on Tubercle Bacilli.—It has recently been shown that the minced tissue of chick embryos facilitates the

growth of tubercle bacilli in synthetic media. The present publication describes the effect of this extract on the rate of growth, morphology and virulence of tubercle bacilli cultivated in the media recently developed by Dubos and Middlebrook. The extract (CEE) was made from 11 day old chick embryos and most of the experiments were carried out with human strains of tubercle bacilli, H37Ra (avirulent) and H37Rv (virulent). From 0.5 to 1.0 per cent of CEE definitely enhanced the growth of tubercle bacilli in the base medium to which oleic acid had been added. It was also noted that the maximum yield of bacillary growth was reached earlier with the extract than without. The addition of CEE to the oleic acid medium caused the bacilli to organize themselves in serpentine cords which were longer and more dense than those formed in the absence of the extract. The avirulent variants normally grow in irregular clumps. A more striking effect was observed when Tween 80 was added to the base medium. The lipase present in CEE destroyed the Tween 80 and prevented the latter from inhibiting cord formation when virulent strains of bacilli were used. This unspecific effect was fundamentally different from the one obtained when oleic acid was employed. Inoculation experiments with Swiss albino mice seem to indicate that the virulence of H37Rv was slightly increased following cultivation on a medium containing CEE. However, the extract did not confer virulence on the avirulent variant. Extracts prepared from rabbit testicles, from mouse brain, liver and spleen, from leukocytes of man and rabbits, guinea pigs and mice, and from egg yolk failed to show any effect on cord formation although they had some enhancing effect on the growth rate of tubercle bacilli.—*The effect of chick embryo extract on the growth and morphology of tubercle bacilli, Hubert Bloch, J. Exper. Med., September 1, 1948, 88: 355.*—*(J. S. Woolley)*

Sphingomyelin and Growth of Tubercle Bacilli.—In addition to certain long chain fatty acids available as Tween 60, Tween 80, et cetera, there exist other lipids, of tissue origin, which have been shown to enhance the growth of tubercle bacilli. The growth-promoting effect of various sphingomyelins, cerebrons and phrenosin was studied by adding them to a basal asparagine medium similar to the author's original medium. Their effect on the growth of small numbers of tubercle bacilli (H37Rv) was studied when the nonionic wetting agent Triton A20 was used instead of Tween 80. The former is a heat-stable arylalkyl polyether of phenol which disperses the cultures without increasing the growth. All the preparations of sphingomyelin from kidney, liver, brain and lung proved almost as effective as serum albumin in enhancing the yield of bacilli. The cerebrosides and the phrenosin, on the other hand, decreased the ability of the medium to support the growth of small inocula. The wetting agent Triton A20 increased the yield through its dispersing effect on the lipids. The neutralizing effect of sphingomyelin on the toxicity of long chain fatty acids was similar to that of serum albumin. Sphingomyelin is a diaminophospholipid in which lignoceric acid is combined in amide linkage with the base sphingosine, phosphocholine being esterified to the latter. Lingoceric acid and its amide also enhanced growth; sphingosine did not. Sphingomyelin, like serum albumin, protects the bacilli against the toxic effects of long chain fatty acids and also supplies a water-dispersible source of lignoceric acid which is available for metabolic utilization.—*The effect of sphingomyelin on the growth of tubercle bacilli, R. J. Dubos, J. Exper. Med., July 1, 1948, 88: 73.*—*(J. S. Woolley)*

Wetting Agents and Growth of Tubercle Bacilli.—It is possible to obtain finely dispersed, submerged growth of tubercle bacilli by adding to the medium certain nonionic wetting agents. The polyoxyethylene esters of oleic acid (Tween 80, Tween 40, et cetera) have proved especially effective in this respect. Unfortunately, the ester linkage in these wetting agents is susceptible to enzymatic hydrolysis by lipases, a fact which prohibits

their use in media containing animal tissues or fluid rich in these enzymes. What is needed is a wetting agent capable of promoting dispersed growth of tubercle bacilli yet stable in the presence of animal tissues. Triton A20, an arylalkyl polyether of phenol, in some respects fulfills these requirements and experiments are reported in which this agent is compared with the most used of the oleic acid esters, namely, Tween 80. Triton A20 is miscible in all proportions with water and can be handled like Tween 80 in the preparation of liquid and agar media. By adding various amounts of Tween 80 or Triton A20 to a basal medium containing amino acids, buffer salts and crystalline bovine serum albumin, the effect of these wetting agents on the growth of virulent and avirulent tubercle bacilli could be readily studied. Also the influence of blood serum on the growth-dispersing effect of these agents was observed and finally their combination with either oleic acid, sphingomyelin or crystalline serum albumin was investigated. The following results were noted: Tween 80 loses its ability to disperse cultures of tubercle bacilli in media containing serum; Triton A20 does not. Tween 80 increases the yield of growth, probably by supplying oleic acid to the bacilli; Triton A20 does not. In concentrations sufficient to cause dispersed growth, Tween 80 (purified by removal of unesterified fatty acid) and Triton A20 are completely innocuous to virulent tubercle bacilli. Triton A20, however, exhibits a marked toxic effect on the avirulent variants of mammalian strains. The two wetting agents also differ in their effects on the morphological aspects of the bacterial cultures. Although Triton A20 prevents the formation of large amorphous bacillary clumps, it is less effective than Tween 80 in preventing the formation of long bacillary strands. With Tween 80 these cords of bacilli are not formed, resulting in a better dispersion of the bacilli.—*The effect of wetting agents on the growth of tubercle bacilli, R. J. Dubos & G. Middlebrook, J. Exper. Med., July 1, 1948, 88: 81.—(J. S. Woolley)*

Tuberculous Droplet Infection in Rabbits. II.—The fate of tubercle bacilli inhaled after tuberculosis already had been established by droplet infection has been studied to determine the effects of developing resistance in infected animals. At intervals of two to eleven weeks after normal rabbits had inhaled small numbers of virulent bovine tubercle bacilli in droplet nuclei, groups of these animals received another single exposure during which each animal inhaled about 20,000 separated bacilli. Normal control rabbits receiving this dose died within four weeks. Rabbits reinfected within four weeks of the initial exposure seemed to be about as susceptible to further implantation of separated organisms as were normal animals. However, reinfection tubercles always contained far smaller numbers of bacilli than initial tubercles of the same age. This difference was present even in animals re-exposed at two weeks. When reinfection was delayed five weeks, however, the bacilli of reinfection did not grow sufficiently in most animals to induce new tubercles in the lungs. It is suggested that after five weeks many rabbits become immune to reinfection although, in a few, exposure to massive reinfection seemed to stimulate the progress of the initial infection. It is possible that such reinfection so saturated the defense mechanism of some animals that, although the bacilli of reinfection were destroyed, those in the initial focus increased more rapidly than would be expected. It is also possible that a sensitization reaction may have been present. The basic effect of the acquired resistance of rabbits to tubercle bacilli in these experiments seems to be an inhibition of the multiplication of the bacilli. *Tuberculosis of rabbits induced by droplet nuclei infection: II. Response to reinfection, H. L. Ratcliffe & W. F. Wells, J. Exper. Med., June, 1948, 87: 585.—(J. S. Woolley)*

Tuberculoproteins. II.—Preparations obtained from unheated culture filtrates of tubercle bacilli were sufficiently distinctive according to their sedimentation and diffusion diagrams to warrant an investigation of their

serological identities. Only two serologically different proteins were found in these unheated filtrates. One of them was the protein which had a sedimentation constant of 3.4 S. and the other was in filtrate fractions with a constant of 2 S. The material giving the higher rate was practically homogeneous antigenically and was in a fraction precipitated by one-quarter saturated ammonium sulphate at pH 4.4. The fractions obtained by full saturation contained only the second antigenic entity; fractions obtained by one-half and three-quarters saturation appeared to be mixtures of the two. That these proteins were distinct was demonstrated by three methods: quantitative precipitin and precipitin absorption tests with rabbit antisera, skin tests in guinea pigs actively sensitized with the culture filtrate fractions, and skin tests in passively sensitized guinea pigs. In the guinea pigs sensitized with the first of the two proteins the sensitization was against only the homologous fraction. All fractions proved active for tuberculin-sensitive human beings or tuberculous guinea pigs. A third antigen of unknown nature was found by means of the precipitin test but only in certain fractions from the virulent culture filtrate. The protein with the constant 3.4 S. could not be demonstrated serologically in an Old Tuberculin made from the same culture filtrate as the unheated preparation from the virulent organism.—*The proteins in unheated culture filtrates of human tubercle bacilli II. Determination of serological properties, J. R. McCarter & E. B. Bevilacqua, J. Exper. Med., March, 1948, 87: 245.*—(*J. S. Woolley*)

Complement Fixation in Psittacosis.—The serum complement fixation reaction, supplemented by clinical and epidemiologic data, furnished the basis for a diagnosis of psittacosis in 21 cases, in none of which was the disease fatal. A fourfold or greater rise in antibody titer during the course of the illness occurred in the serum of 9 persons, from one of whom psittacosis virus was isolated. Serum from 12 persons had a significant titer (1 to 32) when it was first secured ten days or later after the onset of the disease. All the patients had manifested the clinical characteristics of the disease. However, pulmonary lesions were not demonstrated by roentgenograms of the chest in 4 of 16 persons examined. All the patients reported intimate contact with birds, chiefly pet parakeets and pigeons. The serum complement fixation reaction became positive about the seventh to the tenth day of the disease, achieved a maximum titer about the fifteenth to the thirtieth day and persisted, though in low titer, as long as the serum was tested.—*Complement fixation reactions in psittacosis, D. J. Davis, Arch. Int. Med., May, 1948, 81: 623.*—(*G. C. Leiner*)

Humidity and Air-borne Bacteria.—An understanding of the influence of atmospheric conditions on the viability of air-borne microorganisms is essential to any attempt to formulate the epidemiology of air-borne infections. In the present study bacterial suspensions were atomized into an experimental chamber under controlled conditions of temperature and relative humidity. The most striking fact revealed by these tests was the demonstration of the existence of a narrow range of relative humidity, in the vicinity of 50 per cent, which is rapidly lethal for microorganisms freshly sprayed into the atmosphere from a broth suspension. The ultimate extent of dehydration depends on the relative humidity, since the droplet eventually comes into a condition of equilibrium with the atmosphere. The viability of pneumococcus, Type I, sprayed into the atmosphere from a liquid suspension was measured as a function of the relative humidity. When broth, saliva, or 0.5 per cent saline solution was employed as the suspending medium, a very high mortality rate was observed at relative humidities in the vicinity of 50 per cent. However, at humidities above or below this value the microorganisms survived for long periods. Measurement of the rate of settling of droplets employed in these experiments demonstrated that the disappearance of microorganisms from the air is a true lethal process rather than a manifestation of aerosol collision processes.

When a saline-free fluid was used however, the sharp peak in death rate at intermediate relative humidities disappeared. The lethal effect of intermediate relative humidities on pneumococci atomized from a saline-containing suspension was increased when the particle size of the atomized droplets was increased or when the temperature was raised. Cultures of group C hemolytic streptococcus and staphylococcus sprayed from a broth medium exhibited the same general survival pattern as a function of relative humidity although the mortality rates were smaller than that of the pneumococcus. These effects can be explained by assuming the existence of a critical degree of cellular dehydration at which microorganisms become much more sensitive to toxic agents than in states where either more or less water is bound to the cell.—*The lethal effect of relative humidities on air-borne bacteria, E. W. Dunklin & T. T. Puck, J. Exper. Med., February 1, 1948, 87: 87.*—*(J. S. Woolley)*

Humidity and Influenza Virus.—To study the effect of air moisture on the infectivity of influenza virus, white mice were exposed to atmospheres containing known amounts of atomized influenza A virus (PR8 strain) of constant potency under conditions of varying humidity. It was found that an amount of atomized virus suspension which produced a 100 per cent mortality rate in animals exposed at 30 and 80 per cent relative humidity, respectively, resulted in the death of only 22.5 per cent of mice at a humidity of 50 per cent. The humidities between these values gave intermediate results. The infectivity of the air-borne virus decreased so rapidly at a humidity of 50 per cent that it was impossible to secure a 100 per cent mortality rate in the exposed mice even by greatly increasing the dose of virus atomized. The use of a dialyzed virus suspension at a humidity of 50 per cent resulted in the death of all exposed mice. This suggested that the deleterious influence of humidity was related to the presence of sodium chloride in the atomized suspension. These findings with influenza virus closely resembled those obtained by Dunklin and Puck with pneumococci, streptococci, and staphylococci which would suggest that the factor responsible for the lethal effect of humidity is common to moist particles containing either the above mentioned bacteria or influenza A virus.—*The influence of relative humidity on the infectivity of air-borne influenza A virus (PR8 strain), W. Lester, Jr., J. Exper. Med., September 1, 1948, 88: 361.*—*(J. S. Woolley)*

Pulmonary Edema due to Epinephrine.—The intravenous injection of epinephrine increases the central action of neurotropic substances at least fivefold and in the course of such injections many rabbits develop severe dyspnea. This is followed by pulmonary edema with pink foam and fluid pouring from the nostrils and mouth, convulsive struggling and death. Pulmonary edema can be produced regularly by injecting 5 cc. of water or physiological saline solution with the epinephrine; without the additional fluid, pulmonary edema occurs only sporadically. Combining the epinephrine with depressants like alcohol or paraldehyde prevents the development of pulmonary edema. Angiocardiography was used to study the pathogenesis of this epinephrine pulmonary edema by injecting first epinephrine and then thoratrast into the ear vein of rabbits. Radiographs taken immediately after the injections revealed the jugular veins filled to capacity with the opaque material. The dilated veins could be followed to the right auricle and 40 seconds after the injection no opaque dye was visible in the left heart or aorta. Because of the increased pressure in the pulmonary circulation and the right heart, the injected dye took the route of least resistance and spread backwards into the peripheral tributaries of the jugular veins in the head. No trace of the opaque dye could be seen in the radiographs of the control rabbits. Experimental observations indicate that a sensitized heart may respond abnormally to small amounts of epinephrine. Although large doses are required to produce left ventricular failure and pulmonary edema in normal ani-

mals, small amounts of epinephrine or similar pressor substances may significantly affect a seriously weakened left ventricle in man.—*Angiocardiographic experiments on the pathogenesis of adrenalin pulmonary edema in rabbits, A. Elkeles, Brit. J. Radiol., September, 1948, 21: 472.*—(*L. Hyde*)

Blood Pressure and Respiration.—By means of the cardiac catheterization technique and a Hamilton manometer, pressure tracings were obtained from the right auricle and ventricle of 4 normal subjects, of 6 patients with chronic pulmonary disease, and of 4 patients with auricular fibrillation and heart failure. A study was made of the influence of respiration on the intracardiac pressures. In normal subjects the average maximum pressure in the auricle was 22.5 mm. of water during expiration and 7.5 mm. of water during inspiration; the average minimum pressure was 6.0 mm. of water during expiration and minus 10 mm. of water during inspiration. The average systolic blood pressure in the right ventricle was 27.25 mm. of mercury during expiration and 22.25 mm. of mercury during inspiration; the average diastolic pressure was 5.75 mm. of mercury during expiration and minus 0.5 mm. of mercury during inspiration. In patients with chronic pulmonary disease in the absence of heart failure, only the right ventricular systolic pressure was elevated. Systolic pressure in the right auricle and diastolic pressure in the right ventricle were normal. In patients with chronic pulmonary disease in the presence of heart failure, both systolic and diastolic right ventricular pressures were elevated. Auricular pressures were also elevated because of the hindrance of auricular emptying which resulted from the increased diastolic ventricular pressure. In normal subjects and in patients with chronic pulmonary disease, with or without heart failure, intra-auricular and intraventricular pressures were lower during inspiration and higher during expiration. The respiratory variations of intra-auricular pressure are ascribed to (*1*) variations in the intrapleural pressure and (*2*) action of the elastic fibers of the lungs which, during inspiration, distend the auricular walls. The respiratory variations of intraventricular pressure are ascribed to (*1*) variations in the intrapleural pressure and (*2*) decreased peripheral resistance in the pulmonary circuit during inspiration. In patients with heart failure and auricular fibrillation, maximum and minimum pressures in the right auricle and right ventricle were abnormally elevated and did not change during respiration. The lack of respiratory variation in the pressures in the right auricle and in the right ventricle is ascribed to repletion and distention of the right auricle and to pulmonary stasis, respectively. (Authors' Summary)—*Intracardiac blood pressure in human subjects and its relation to the respiratory phases, A. Battro, H. Bidoggia, E. Pietrafesa & F. E. Labourt, Am. Heart J., January, 1949, 37: 11.*—(*G. C. Leiner*)

Artificial Respiration.—Artificial respiration was produced in the cat, dog, monkey and rabbit by electrical stimulation of one or both phrenic nerves. Adequate minute volumes of respiration, arterial blood oxygenation and elimination of carbon dioxide were achieved in the absence of spontaneous respiration by submaximal stimulation of one phrenic nerve. A severalfold increase in ventilation was seen with bilateral phrenic nerve stimulation. Tidal volume, minute volume and, therefore, arterial oxygen and carbon dioxide tensions, can be regulated by adjustment of the peak voltage used for the stimulation. Artificial respiration was maintained as long as twenty-two hours.—*Electrophrenic respiration, S. J. Sarnoff, Esther Hardenbergh & J. L. Whittenberger, Am. J. Physiol., October 2, 1948, 155: 1.*—(*G. C. Leiner*)

Pulmonary Capillary Pressure.—A venous catheter was introduced into the pulmonary artery of dogs and wedged into a distal ramification so as to obstruct its lumen. Pressures beyond the point of obstruction were recorded through a hole in the tip of the catheter with a Hamilton manometer and with

a saline column. An arterial catheter was introduced by way of the carotid artery and left side of the heart into a pulmonary vein in similar fashion and pressures were similarly recorded. In dogs, the pressures recorded with a Hamilton manometer through the catheter blocking the pulmonary artery varied between 5 and 8 with an average of 6 mm. of mercury while those measured simultaneously through the catheter blocking the pulmonary vein varied between 6 and 14 with an average of 10 mm. of mercury. The former is probably lower than true pulmonary capillary pressure by the "velocity factor" and the latter too high by the same factor. It is suggested that the true mean capillary pressure with unobstructed flow normally lies between these two figures, the average in 6 animals varying from 5.5 to 10 mm. of mercury with an average of 8.3 mm. of mercury. It is considered that damage to the left side of the heart from arterial catheterization prohibits its use in man. (Authors' Summary)—*Pulmonary capillary pressure in animals estimated by venous and arterial catherization, H. K. Hellems, Florence W. Haynes, L. Dexter & Th. D. Kinney, Am. J. Physiol., October, 1948, 55: 99.—(G. C. Leiner)*

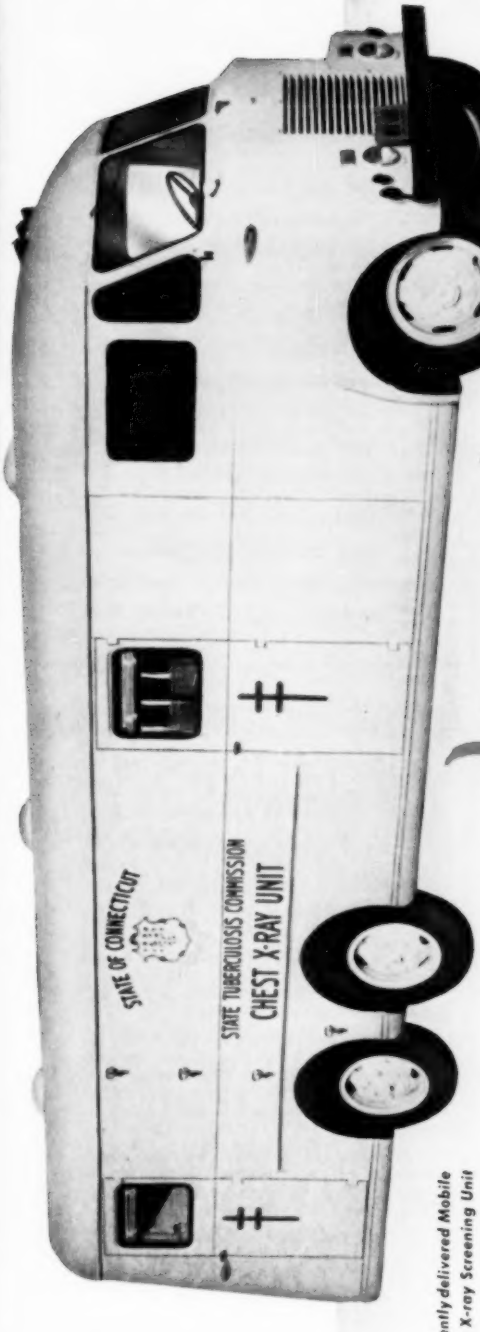
Joint Reflexes and Respiration.—Passive flexion of the hind limbs of cats and dogs is accompanied by variable changes in rate and depth of respiration. These changes do not appear to result from the stimulation of receptors in the knee joint. Other types of stimuli, including those secondarily induced by the flexion, such as movements of the trunk, were as effective in stimulating respiration. Similar changes occurred in dogs whose knee joints had been partially denervated. Electric stimulation of nerves to the knee joint was effective only at intensities great enough to activate slower conducting fibers, resulting in what appeared to be pain hyperpnea. It is concluded that, under the conditions of anesthesia used, joint reflexes do not play a significant role in the production of the respiratory changes during passive flexion. It is also concluded that there is no satisfactory evidence which indicates that limb reflexes are important in the regulation of respiration during exercise in man. (Authors' summary).—*Joint reflexes and regulation of respiration during exercise, E. Gardner & J. Jacobs, Am. J. Physiol., June, 1948, 153: 567.—(G. C. Leiner)*

Public Health and Epidemiology

Prevention of Tuberculosis.—Dr. Wyndham E. B. Lloyd, tuberculosis officer for South Devon, has emphasized recently that tuberculosis is not only a disease but a social and economic problem. To prevent infection the first step is to discover the individuals who harbor the disease. When the patients have been found, they must be treated and educated so that they are no longer a danger to others. This is especially true since the main reservoir of infection is the person whose sputum contains tubercle bacilli but who does not know or care; one of the most common is the middle-aged person who has been diagnosed as having "bronchitis." To prevent the spread of tuberculosis through milk, all dairy herds should be tuberculin-tested and, if the herds are untested, milk should be heat-treated. Since the establishment of the National Health Insurance Service in 1911, patients in the British Isles are sent by their own doctors to tuberculosis officers. Contacts then are called in for examination. Mass miniature roentgenography is now also available. Too much stress cannot be laid on environment in the prevention of tuberculosis. The important factors include adequate housing, water supply, proper ventilation, and absence of overcrowding. Children must have sufficient diet and "there must be freedom from want, from fear, and from insecurity." Nothing is gained by sending a patient from a sanatorium back to overcrowded slum conditions. At first sight, the results of the work done in combating tuberculosis seem very unsatisfactory. Infections seem to be increasing; the total number of cases of the disease is greater; the waiting lists for sanatoriums are long. However, notifications are no indication of the increase of the disease, for the more the search,

the greater the number of cases that will be found. Between 1914 and 1943 the crude death rate for pulmonary tuberculosis has been reduced by 50%. What is most needed, Doctor Lloyd concluded, is an enlightened public.—*Prevention of tuberculosis: Environmental factors (Editorial), Brit. M. J., November 13, 1948, 4584: 870.*—(*R. W. Clarke*)

Photofluorography.—Experience in Zürich, Switzerland, shows that a case finding program by means of photofluorography is not only financially possible but profitable. Of 16,585 adults examined, 74 per cent were normal, 19.9 per cent had unimportant abnormalities and 6.1 per cent were judged to be in need of further investigation. Of these, 1.56 per cent needed either supervision or treatment for tuberculosis; 0.76 of the 1.56 per cent were previously unrecognized. Previously unrecognized infectious tuberculosis was present in 0.16 per cent. The authors calculate that the initial cost of the program and the operative expenses over two years (1945 and 1946) have already been saved the public if one assumes that each of the 24 infectious individuals discovered would be responsible for 3 or 4 new cases if allowed to go undetected. This program should prove even more profitable as the initial cost of the equipment is distributed over more than a two year period.—*Abklärungsergebnisse und Wirtschaftlichkeit des Schirmbildverfahrens in der Stadt Zürich, H. O. Pfister & W. Schwarz, Schweiz. med. Wchnschr., January 31, 1948, 78: 87.*—(*H. Marcus*)



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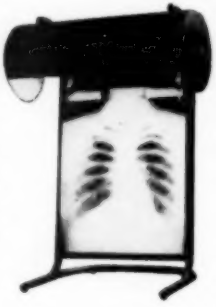

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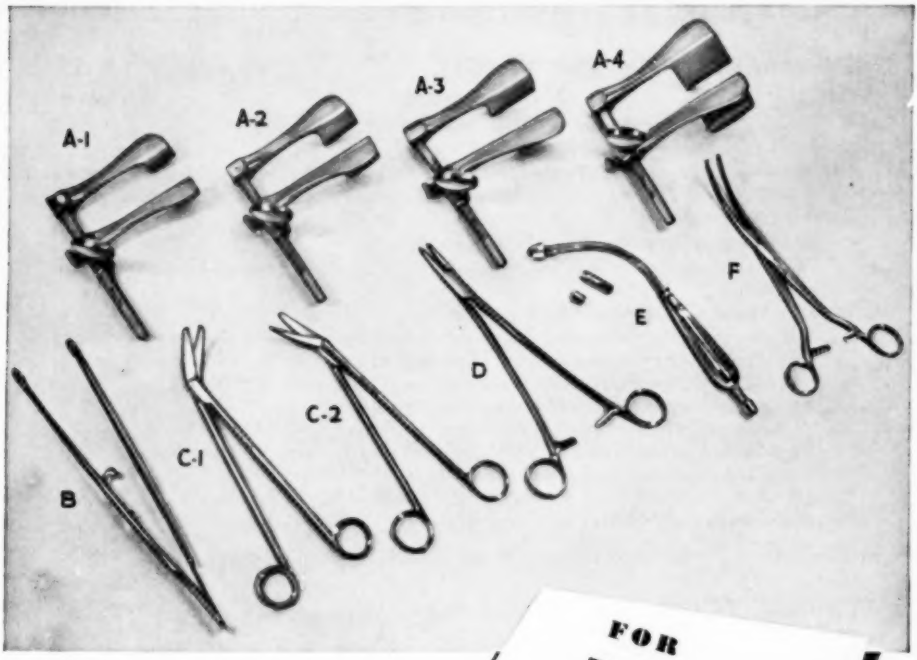

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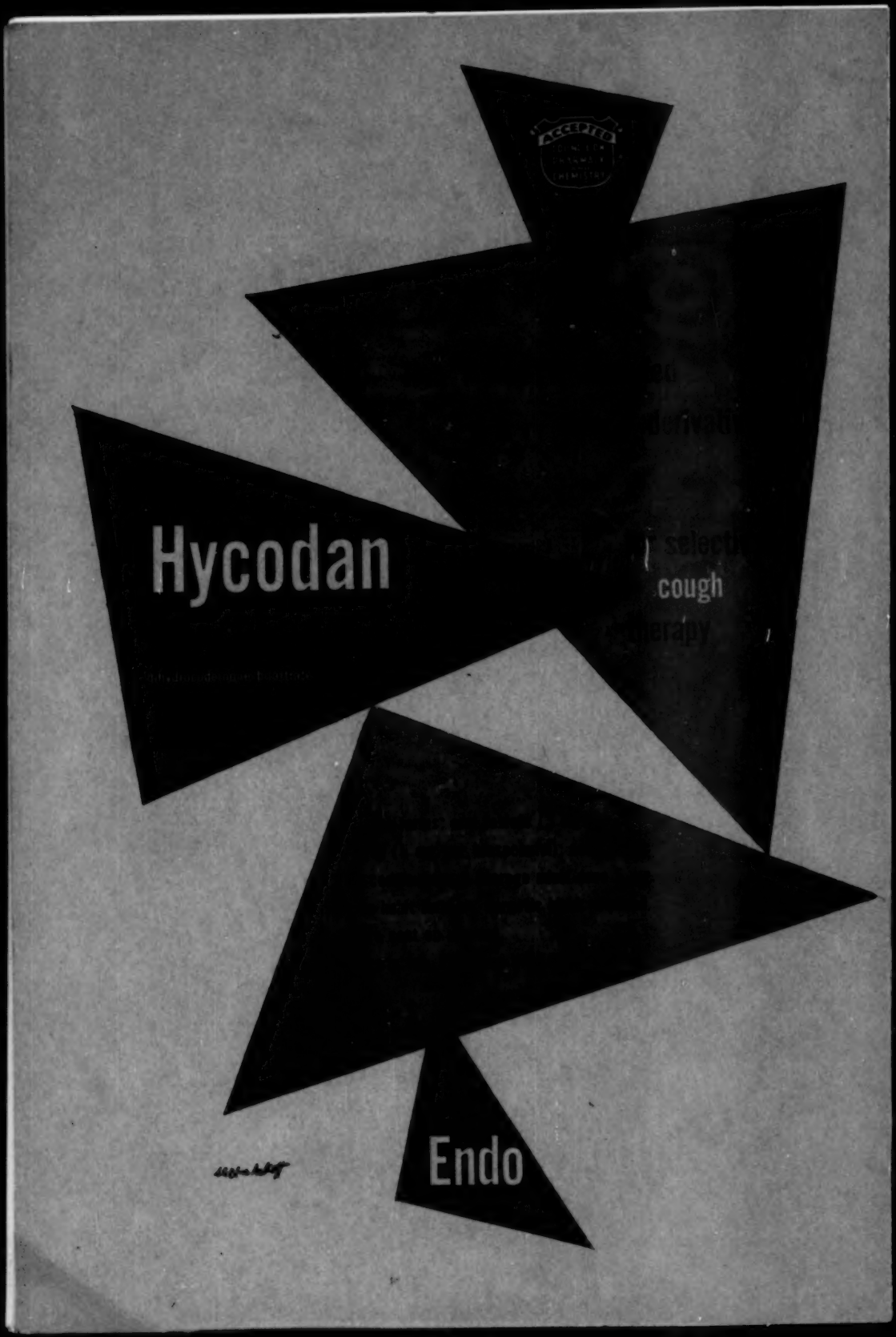
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